

A COMPARATIVE STUDY  
OF  
THE MEASLES AND CANINE DISTEMPER  
VIRUSES.

A Thesis Presented for the degree

of

Doctor of Philosophy

by

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## HISTORICAL INTRODUCTION

### Measles

Measles is a disease of great antiquity; it has been known throughout the centuries, and has retained all its characteristics, uninfluenced by environmental, ecological or therapeutic factors. It is perhaps the most highly infectious of all known exanthemata. Almost everybody has suffered from measles, and it is "as inescapable and as inevitable as death and taxes". (Gibson and Gordon, 1954). Toronta K. Jerome has compared this to love, because the "later one gets it, the more serious it is". Usually a self limited mild disease, it can be serious in the very young, and the very weak. It has often been regarded as "an invitation to secondary infections in badly nursed or ill cared for patients".

## INTRODUCTION

The term measles was probably derived from the Latin word "misellus", meaning miserable, a term common to leprosy and various other skin eruptions and sores. John Haderic, in the 12th Century, first used this term in Great Britain. For a thousand years, after the days of Hippocrates, the physicians of Greece, Rome and Arabia (A.D. 650) failed to distinguish measles from

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The term measles was probably derived from the latin word "Misellus", meaning miserable, a term common to leprosy and various other skin eruptions and sores. John Gaddeston, in the 12th Century, first used this term in Great Britain. For a thousand years, after the days of Hippocrates, the physicians of Greece, Rome and Arabia (Yel Yehudi A.D. 68) failed to distinguish measles from



small-pox.

The first physician ever to distinguish the two, was the Persian, Abu Bakr Muhammed Ibu Zakurriya otherwise Rhazes in about 970 A.D. According to his teaching, measles was caused, by the "vehement ebullition of bile in the blood" and the small-pox was due to "heat and putrefaction in the fermented blood". At about the same time the Israelite Isaac said it was due to "Viciousness of the menstrual blood". The noxious part of the pregnant woman's blood passed into the foetus, and the child got rid of this by the characteristic haemorrhagic rashes - Measles. This view was accepted by some physicians till as late as 1660 A.D. (Thomas Willis).

Historically, measles has played no inconsiderable part in shaping the fate of countries, especially in Europe and America. As for example, in 1871 during the Siege of Paris, when the ease of the German armies victory was due to the fact that fifty per cent of the French troops were ill with measles. (Babbot and Gordon, 1954). In the battle between Paraguayans and Brazilians in 1865, more than a fifth of the Paraguayan army was disabled with measles.

Although it was Rhazes who recognised that measles and small-pox were separate diseases, it was Sydenham (Rolleston 1924) who made it possible to define the clinical

pictures of the two exanthemata. His minute observations of the London epidemics of 1670 and 1674, and his accurate and beautifully written records of the clinical features of the disease, give the first sound exposition of the natural history of measles.

The clinical picture of measles is one of the clearest of all viral diseases, and the diagnosis can often be made with certainty on clinical grounds alone. A lifelong immunity follows and an effective level of antibodies seems to be maintained without further apparent or inapparent attacks. That this immunity persists for as long as sixty-five years, is witnessed by Panum's observations on the epidemic which occurred in the Faroe Islands in 1846. Second attacks of measles are virtually unknown (Burnett, 1959).

The contagiousness of measles was generally recognised, but it was not until 1758 that any attempt was made to establish this fact experimentally. Dr. Francis Home (1759), an Edinburgh physician, deliberately attempted to transmit measles from person to person in an effort "to render this disease more mild and safe". His method was to saturate cotton swabs with the blood, "that magazine of all epidemic diseases", from "cutaneous veins" obtained by "superficial incisions amongst the thickest of measles".

This swab was often carried in his "pocket note-book" though he kept it moist. This he applied and allowed to remain in contact for as long as three days in an incision made in the skin, after the recipient has been bled for a quarter of an hour. Subjects for the experiment ranged from 7 month old babies to thirteen year old children. The storage of the blood soaked cotton swab varied from two days to five weeks. He claimed to have produced the "milder disease" in ten out of fifteen experiments, the incubation period varying from 6 to 11 days. He also tried, three times unsuccessfully to reproduce the disease by using nasal swabs taken from measles patients and introducing them into the nostrils of susceptible hosts.

Although this is not the actual method of spread in nature, Home had established that the infective agent was present in the circulating blood of patients ill with measles.

Almost a hundred years were to pass before the next advance came in our understanding of measles. In 1846, a young Danish physician, Pater Ludwig Panum, was sent by his government to deal with an epidemic of measles which was raging in the Faroe Islands. This epidemic was remarkable because these isolated islands had been free of measles for 65 years and almost the whole population of

7,782 people developed the disease; only 98 elderly people, all over the age of 65, escaped the infection. Panum made full use of this unique opportunity to investigate the epidemiology of measles and his report of 1847, and later papers on the same subject, won for their author a high reputation. Together with Budd's work on Typhoid Fever (1859) and Snow's studies on Cholera (1849), Panum's contributions formed the observations on which the modern science of epidemiology is founded. It is to be noted that these three pioneers in epidemiology all reached their conclusions about the contagiousness of diseases without any knowledge of the agents causing them.

Panum, by questioning and clinical observation, was able to determine that measles spread from individual to individual and from village to village. He traced the sources of the epidemic to a carpenter who had travelled to the islands from Copenhagen several days after being in contact with friends who were victims of measles. He accumulated evidence that the disease was only transmissible from patients during the pre-eruptive and early phases of the illness and he observed that, after an attack, an immunity developed which was of life-long duration. His clinical observations enabled him to fix the incubation period



accurately at 13 - 14 days. As a preventive measure he advocated the isolation of patients and their contacts and the cessation of communications between one village and the next.

In 1898 Josias claimed to have transmitted the disease for the first time to an experimental animal, namely a monkey. Then followed a period between 1910 and 1954 when a number of workers attempted to transmit the infection to a wide variety of hosts. Monkeys, rabbits, mice, guinea-pigs, hamsters, chick embryos were all used, but none with any real success.

The classical work of Home, already referred to, was further pursued by Hektoen (1905). He was able to infect two individuals, by inoculating whole blood drawn from measles patients, 24 and 30 hours after the appearance of rash. Typical measles developed after an interval of 11 and 13 days. Papp (1937) went further to show that it was leucocytes in blood that were responsible for conveying the infective agent. The first attempt to prove the viral nature of the infective agent was by Goldberger and Anderson (1911). They were able to infect monkeys with the bacteria-free filtrates taken from measles patients. It is of interest to speculate whether the experiments, conducted six years



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earlier by Carre (1905) in which distemper was similarly transmitted, was the incentive for Goldberger and Anderson's work. Leucas et al (1912) repeated and confirmed the findings and thus the viral nature of the disease was established.

The other big gap in understanding of the natural history of the disease was the route of transmission. A suspicion entertained by Panum was confirmed by the experiments of Blake and Trask (1921), who obtained the pharyngeal washings from patients 6 days before, and surprisingly as many as 22 days after the appearance of the rash, and introduced them directly into the trachea of monkeys. These animals developed a disease "closely resembling human measles", thus proving that infection can take place through air passages.

It is now realised that monkeys, unless specially selected, are not the ideal experimental animals. Ruckle (1957 and 1962) showed that a high proportion of imported monkeys carry a virus which she named the Monkey Intranuclear Inclusion Agent (MINIA). This agent is quite indistinguishable from the human measles virus and is unrelated to any of the simian viruses (foamy agents). Monkeys examined within a few days of capture do not have measles

antibodies in their blood but, in captivity, they rapidly acquire complement fixing antibodies so that eight weeks after capture, 100 per cent of the animals have been infected, (Meyer et al 1962). This evidence suggests that rhesus monkeys are free of measles in their natural habitat and that they acquire the infection after capture from human contacts; large holding compounds for stocks of monkeys awaiting transportation or experimentation would provide ideal conditions for the spread of measles.

Though many other claims have been made to have transmitted the infection to other animals from blood and throat washings of measles patients, the outstanding landmark in the history of measles came when Enders and Peebles (1954) were able to cultivate the virus in tissue culture.

#### CANINE DISTEMPER

Dog distemper is spread as widely throughout the world as measles. Although it has been recognised for more than two centuries, there are but few descriptions of it before the middle of the 17th century.

Perhaps the first person to give an accurate description of the canine distemper was Ulloa (1735-1746). He

found the disease to be common in the dogs he encountered in his travels through South America. According to Henning (1956) the disease was reported in Spain in 1761, and it probably spread to Europe from Asia and Peru and then to Great Britain via France. The canine population of Greenland was first affected in the year 1929 (Shroeder 1929).

It is a disease of protean character with very variable symptoms. Little was understood about it and, according to Blaine (1832), Darwin called it a "debilitating catarrh". Very often it was confused with rabies. In 1809, that remarkable physician, Edward Jenner, who had earlier introduced vaccination against smallpox, turned his trained mind to observe and record the precise differences between these two diseases "in the hope to ward off its consequences by vaccination". Jenner believed that canine distemper had been imported recently into Great Britain. "I haven't been able to trace it much beyond the middle of the last century", he says, "but it has since spread rapidly".

Blaine (1832) in his book on canine pathology, also confirms that this disease "does not appear to have been known a century ago". He described it as an occasional epidemic which visits different countries every 3 or 4 years, than as a "settled constitutional among dogs, like measles

or whooping-cough in the human".

At first the cause was thought to be a bacterium. Copeman (1900), Lignieres (1903), Phisalix (1903), Ferry (1911), McGowan (1911), Torrey and Rahe (1913), all held the view that the organism known now as Bordetella bronchiseptica was the aetiological agent. It was possible to produce a distemperlike illness in dogs by inoculating this bacterium but at no stage of illness could the agent of distemper be recovered from the bloodstream.

A viral aetiology was, however, postulated by Carre (1905), Lignier~~s~~ (1906), Hardenbergh (1926), and Puntoni (1923 and 1924); Carre used a bacterium-free filtrate to infect susceptible animals, and Hardenbergh showed that dogs which had recovered from experimental infection with Bord. bronchiseptica were still susceptible to distemper when infected with a bacterium-free filtrate.

These early observations were confirmed and the viral nature of distemper was established by the classical work of Laidlaw and Dunkin (1926 ~~and~~ 1928). Using ferrets and dogs from clean and uninfected stock they were able to transmit the disease from dog to dog, dog to ferret, ferret to ferret, and from the ferret back again to the dog. They also established that infection took place by the inhalation



of infected material, and showed that the causative agent could not be cultivated on artificial culture media. This agent passed through Pasteur-Chamberland L2, Mandler, and membrane filters rather less readily than the foot and mouth disease virus, a fact which led them to suspect that it was somewhat larger in size.

are mainly catarrhal in nature: mild fever, with coryza, conjunctivitis and photophobia are characteristic and the appearance of Koplik spots on the buccal mucosa is diagnostic. An evanescent erythematous rash may occur at this time. On the 12th - 13th day, but almost always on the 14th day a macular or maculo-papular rash appears first on the forehead, face and neck, spreading rapidly to the trunk and limbs, and is accompanied by heightened fever. Occasionally the rash is hemorrhagic (in thrombocytopenic subjects) and the disease assumes great severity. The virus is present in the blood and in the nasopharyngeal secretions during the prodromal period, and it persists for about 2 days after the appearance of the rash.

The most serious complication is encephalomyelitis, it carries with it a case mortality which may be as high as 10 per cent. Although some authorities state that the incidence of this complication is about one in 10,000 (Rake



A COMPARISON OF THE CLINICAL  
PICTURES OF MEASLES AND CANINE DISTEMPER

MEASLES

In measles the prodromal signs and symptoms appear 7 - 10 days after exposure and are mainly catarrhal in nature; mild fever, with coryza, conjunctivitis and photophobia are characteristic and the appearance of Kopliks spots on the buccal mucosa is diagnostic. An evanescent erythematous rash may occur at this time. On the 12th - 19th day, but almost always on the 14th day a macular or maculo-papular rash appears first on the forehead, face and neck, spreading rapidly to the trunk and limbs, and is accompanied by heightened fever. Occasionally the rash is haemorrhagic (in thrombocytopaenic subjects) and the disease assumes great severity. The virus is present in the blood and in the nasopharyngeal secretions during the prodromal period, and it persists for about 2 days after the appearance of the rash.

The most serious complication is encephalomyelitis; it carries with it a case mortality which may be as high as 38 per cent. Although some authorities state that the incidence of this complication is about one in 10,000 (Rake

1959) cases, others give a much higher figure. Thus, in Chicago, Gibbs et al (1959) reported that 5.2 per cent (37 cases) of 717 patients developed "clinically evident encephalitis". Furthermore, no less than 51 per cent of the remaining 680 patients showed abnormal electro-encephalograph tracings despite the fact that none had clinical evidence of encephalitis. Although in the great majority the tracings returned to normal, it was found in two cases that the abnormal tracings increased, culminating in convulsions. In one of these two cases, the family doctor diagnosed "idiopathic epilepsy". In three other cases where encephalographic tracings returned slowly to normal, intellectual deterioration and behaviour disorders were subsequently reported. It was three year old children particularly who appeared to be involved and 72 per cent of this age group were described as having abnormal encephalographs and in 25 per cent, the tracings were "extremely abnormal".

These observations are obviously of great importance and suggest that the measles virus, and perhaps other viruses too, are related causally to a proportion of cases of epilepsy and mental retardation; they merit further investigation.

It is more than probable that, during the generalised spread of the infection in the viraemic phase of measles, not only the central nervous system but many other tissues are involved. Thus, Goldfield et al (1955) found transient electrocardiographic changes in some 19 per cent of patients: changes which could not be attributed to any cause other than the viral infection itself.

Bacterial complications, such as otitis media, and bronchopneumonia, which were so common in the past, have now virtually disappeared with the advent of antibiotics. A pure viral type of pneumonia, with extensive giant cell infiltration of the lung-parenchyma has, however, been described by Moore and Gross (1930), Corbett (1945).

#### CANINE DISTEMPER

In canine distemper the clinical picture is by no means as clear cut as in measles, and the diagnosis may be very difficult. In experimentally infected cases the incubation period is 4 - 6 days. The prodromal signs are largely catarrhal, anorexia and fever are accompanied by mucoid nasal and conjunctival discharges. An evanescent macular rash can occasionally be seen on the inner aspect of thighs and bellies of young puppies. This may be followed

by a decline of the fever. A secondary rise of temperature often follows within 48 hours, and the nasal and conjunctival discharges become frankly purulent. Vomiting and a foetid diarrhoea are present and death due to dehydration is common. The disease, however, may assume a variety of clinical forms and the involvement of the central nervous system is extremely common.

Briefly, the clinical forms of the disease are:-

1. Catarrhal - may be severe, mild or inapparent (The Respiratory Response).
2. Gastro-intestinal - usually severe - (alimentary tract response). Laidlaw and Dunkin (1926 and 1927)  
Slanetz and Smetana (1937)  
MacIntyre et al (1948)<sup>a</sup>.  
Verlinde (1946-48).
3. Nervous. Chorea, epileptiform seizures and fits, encephalitis often associated with hyperkeratosis. (Hard pad disease).

There are many intermediate forms of the disease, which do not fall precisely into any of these descriptions. There has been considerable argument as to whether all these disease processes are caused by one and the same virus, especially with regard to hardpad disease which MacIntyre et al (1948)<sup>a</sup> considered as a separate entity due to a virus immunologically distinct from distemper virus. It is, however, clear from the work of Larin (1954) that the behaviour



of distemper virus isolated from dead and moribund dogs is extremely variable, especially with regard to virulence to ferrets. In a study of 55 different isolates in Great Britain, Larin and Hodgman (1954) found three separate immunological groups with no cross immunity between them. The virus strains varied not only on primary isolation, but also during laboratory study. Larin was of the opinion that "there is progressive and continuous change occurring in the antigenic structure of the distemper virus, which cannot be regarded as a stable entity". The ease with which the canine distemper virus can be adapted to suckling mice, suckling hamsters, chick embryos, and tissue cultures of renal, testicular, and pulmonary canine epithelial cells, would also support the suggestion that the distemper virus can vary readily from the normal. Although the question has not been definitely settled, it is possible that, according to MANSI (1961), hardpad disease is an unusual manifestation of distemper seen in dogs that are either partially immune or when the disease is mild or prolonged.



## THE EPIDEMIOLOGY OF MEASLES AND DISTEMPER

It is agreed by practitioners of medicine, in both human and veterinary fields, that measles and canine distemper are amongst the most highly infectious diseases known. Both diseases are widespread throughout the world and in both the highest incidence of infection is in the young.

### MEASLES

The epidemiological pattern of measles has been well studied in the past and the data have not, so far, been confused by the use of prophylactic vaccines. It is evident, that the main source of infection is the infected patient, in the prodromal catarrhal stage and during the first two days of the eruptive stage. Infection commonly takes place by the inhalation of droplets, but infected fomites may also play a part. Papp (1956) has demonstrated that infection can occur by conjunctival route as well. The greatest number of cases of measles occur in the age group 3 - 5 years and by the age of 20 years, 90 per cent have suffered from the disease. Of recent years, improved child health, the use of antibiotics to control secondary infections and the greater care of patients under the age of three years have all combined to produce a dramatic diminution in the mortality of measles. In a virgin population however, the impact of measles may

be dramatic. Cristensen et al (1952) have described an epidemic in Southern Greenland in which only 5 individuals, out of a total population of 4,262 escaped the disease. The mortality was 18 per cent and was highest in the older age groups. Elkin (1961) mentions the 1901 epidemic of measles in Kolyma, a Russian town, which appeared after an absence of 49 years, with a hundred per cent morbidity and 7 per cent case mortality.

A striking characteristic of measles in Britain is its periodicity, usually the interval between the epidemics is approximately two years, the greatest incidence being in November to March. (Butler, 1946). This is largely determined by accumulation of the susceptible population i. e. the school-going children. Cruickshank (1961) (in agreement with Reid (1958)), is of the opinion that there is a positive correlation between the spread of Streptococcus salivarius and the secondary attack rate of measles, and he deduces that the "aerial dissemination of infective droplets during singing, and talking is important in the spread of measles".

Recent advances in serological techniques have made possible the studies of the pattern of neutralising and complement fixing antibodies in the population. Only about 10 per cent of adults were devoid of antibody (Warren and Cutchins

1957). Children and adults who had a definite history of measles gave positive tests in 98 per cent of cases, but 12 out of 37 cases who denied having had the disease also gave positive results. From this, one can conclude that asymptomatic infections do occur.

### CANINE DISTEMPER

The epidemiology of canine distemper is by contrast with measles, little understood, and the author has not been able to discover in the literature any authoritative studies on the subject. Distemper is known to be especially common in cities and epidemics of it are well known in large kennels, dog-homes and mink and fox farms. Wolves, ferrets, weasles, ermine and martens may also be infected. Epidemics are known to occur following the gathering of many dogs at shows.

Puppies born of immune bitches have a passive immunity, through the colostrum and this protects them until they have been weaned (Hagan and Bruner 1957). Thereafter the puppies are extremely susceptible and in the absence of artificial immunisation infection is highly probable. Dogs bred in isolation, however, in such places as remote and isolated areas and islands may entirely escape distemper. Transmission from clinical and subclinical cases may be by

droplets, or it may be truly air-borne as was believed by Dunkin and Laidlaw (1926). It is possible, but not yet established that, since many Virus inclusions are present in the epithelial cells of the Urinary bladder, the infective agent is shed in the urine along with desquamated infected epithelial cells. If this should be the case then the sniffing of urine from an infected animal would be a second important route of infection, especially in the cold countries and during winter months.

Active immunisation of dogs with anti-distemper vaccines was first introduced by Laidlaw and Dunkin in 1928. Green's (1939) distemperoid vaccine was in general use until 1957 since when the egg adapted strains of Haig (1948) Cabasso and Fox (1949), have been used on a very large scale throughout the world, with the result that the epidemiological assessment of the disease has become a matter of great difficulty. Furthermore, the many varied forms of the clinical picture of distemper and the close resemblance of some of them to canine hepatitis, a disease of completely different aetiology, has confused the position badly. To add to this confusion Bateman (1954) has shown that only about 43 per cent of vaccinated dogs are completely protected. Until full facilities for the accurate virological



diagnosis of canine distemper and hepatitis are available and are used by practising veterinary surgeons the epidemiology of these two diseases is likely to remain ill understood.

#### PRIMATE TISSUES

In 1954 Enders and Peabody with their experience of growing poliomyelitis viruses in non-neural human tissues, were the first to grow measles virus in tissue culture of human post-natal tissues. Whole blood or throat washings collected within 24 hours of the appearance of the rash were used to infect growing human kidney cells. A highly characteristic cytopathic effect was observed 3-10 days later; multinucleate giant cells appeared with acidophilic intranuclear inclusion bodies and masses of eosinophilic material were present in the cytoplasm. The extension of this effect on the cells resulted in the formation of large syncytial masses and finally caused the disruption of the whole cell sheet. They were able to isolate a total of 9 strains, 6 from blood, 2 from throat washings and one from lungs of a case just died during the acute phase. This work was confirmed by Cohen *et al* (1955), and Rucke (1957). Rucke (1957) using human kidney, human amnion and monkey kidney tissue cultures, was able to isolate a total of five strains. The blood gave positive results in all the three cell cultures

## CULTIVATION OF THE VIRUSES

### MEASLES VIRUS

#### PRIMATE TISSUES

In 1954 Enders and Peebles with their experience of growing poliomyelitis viruses in non-neural human tissues, were the first to grow measles virus in tissue culture of human post-natal tissues. Whole blood or throat washings collected within 24 hours of the appearance of the rash were used to infect growing human kidney cells. A highly characteristic cytopathic effect was observed 4-10 days later; multinucleate giant cells appeared with acidophilic intranuclear inclusion bodies and masses of eosinophilic material were present in the cytoplasm. The extension of this effect on the cells resulted in the formation of large syncytial masses and finally caused the disruption of the whole cell sheet. They were able to isolate a total of 9 strains, 6 from blood, 2 from throat washings and one from lungs of a case that died during the acute phase. This work was confirmed by Cohen et al (1955), and Ruckle (1957). Ruckle (1957) using human kidney, human amnion and monkey kidney tissue cultures, was able to isolate a total of five strains. The blood gave positive results in all the three cell cultures

and the throat swab material only in human and monkey kidneys. The cytopathic effect was seen as early as three days in the amnion cultures, in 6 days in monkey kidney and 8 days in human kidney. The specimen for inoculation was collected 20 hours before and 6 hours after the appearance of the rash.

A host of other writers, e. g. Peebles et al (1957), Beck and Von Magnus (1958), have also succeeded in isolating the strains in monkey and human renal tissues. Wright (1957) could grow the virus in human infant kidney tissue, and in human chorion as well as in the amnion tissue cultures. Beck and Von Magnus (1957) failed to grow the virus in embryonic lung tissue. In 1958 Girardi and Warren found that a pure line of normal human heart cells were susceptible and gave a comparatively high yield of virus after only 4 to 5 days' incubation. Schwarz et al (1960) found human heart tissue cells were more sensitive for virus isolation than amnion.

#### EXPERIMENTAL ANIMALS

After the work of Goldberger and Anderson, Blake and Trask, Nevin and <sup>Billman</sup> ~~Blake~~ (1921-23) inoculated monkeys with blood of serially passaged rabbits and claimed positive results.

## Rodents

### a. Rabbits

Rabbits were first used by Harde (1921) as experimental animals and he claimed to have observed rashes on the shaven skin, after administering citrated blood intravenously. Nevin and Bittman (1921 and 1923) observed not only rashes but Koplik spots as well. Grund (1922) had only a partial success, for only 50 per cent of his rabbits were susceptible. Those examined after recovery were not immunised. Duale and D'Aunoy (1922) claimed their rabbits developed all the signs, namely, exanthemata, Koplik Spots, leucopenia, pyrexia (103°F) after an incubation period of 3 - 7 days. Further, the causative agent seemed to become virulent and killed the rabbits in the 4th passage.

### b. Mice, Hamsters, Guinea pigs.

Wright (1957) using 8 week old mouse kidney, 5 week old hamster kidney, and embryonic and 5 day old guinea pig kidneys, for tissue cultures, was able to grow the measles virus.

Imagawa and Adams (1958), Arakawa et al (1959) adapted the measles virus to suckling mice. Imagawa and Adams used the Edmonston strain in its 15th passage for intra-cerebral inoculation of 1 - 2 day



old suckling mice; a lethal effect was seen in 9 to 18 days, and was confirmed by neutralisation tests in tissue culture and by further passages in suckling mice. However, the titre of virus yield was low.

Arakawa et al (1959) used infective blood from a measles patient to inoculate the chorio-allantoic membrane of 9 to 10 day old embryonated hens eggs, and incubated these at 35°C for 4 to 5 days. The chorio-allantoic membrane, showing no visible lesions, was harvested, and a 20% emulsion in saline was made. This emulsion was further concentrated 20 times by differential centrifugation and was used as the inoculum for intracerebral inoculation in mice, with lethal results.

#### Avian Tissues

##### a. Chick embryos

Torres and Teixeira, (1935) were the first to attempt to use fertile eggs to grow measles virus on chorioallantoic membrane and Plotz (1938) also claimed to have used this technique successfully. Rake and his co-worker, (1940) used de-fibrinated blood, and throat washings of patients, collected at the first appearance of rash and from the experimental monkeys as well. After ensuring the bacterial sterility, these materials were inoculated into 8 - 16 day

old eggs on the Chorio-allantoic membrane, (C. A. M.); after incubation, averaging 3 to 5 days, 6 successful results, and 4 failures, were obtained. Appearances seen on the C. A. M. ranged from a single central lesion (traumatic) in most, to several discrete white greyish areas, about 2 mm. in diameter in 25 per cent of the inoculated eggs. However, these lesions failed to appear on further passages. These authors used their chick embryo material to reproduce measles in man and monkeys. However, Rake and his colleagues lost the virus after 66 passages. They concluded their experiments with the remarks (a) "It is certain that measles virus can exist and can be propagated in the absence of any visible lesions" (in eggs) and (b) that reasons for their failure to produce an effective vaccine "were not evident nor will they be fully understood until better methods are available for working with the virus in vitro and in the laboratory".

Toyoshima et al (1960) claim that they have been conducting experiments similar to those of Rake et al from 1943 onwards, and that they have developed their "better method" of interference diagnosis. They used the Edmonston strain and their own isolate in F. L. cells (Toyoshima strain).

They inoculated the virus into the chorio-allantoic and amniotic cavities and after 3 to 4 days incubation, introduced mumps virus and tested for the presence or absence of mumps virus by haemagglutination. From these inoculated eggs, both amniotic and allantoic fluids were separately harvested and titrated in tissue culture tubes to detect the measles virus. Only the amniotic fluid gave a positive result. However, their isolation rates in eggs were low - 4 positive out of 56 inoculations, and even those strains were lost after about 10 passages.

b. Chick Embryonic Tissue

Plotz's (1938) work of growing the measles virus in chick embryo tissue culture, using the monkey as the test animal, was later confirmed by Rake et al (1941). Enders (1940), however, failed to obtain convincing evidence that the virus actively multiplied in living chick embryo tissue cultures because no cytopathic effect could be observed.

Later, Enders and his associates (1957, 1959), took up their work again, using the embryonated hen's egg and chick embryo tissue culture, for the production of vaccine against measles. The Edmonston strain of measles virus after 28 passages in human amniotic cell culture was inoculated to the amniotic sac of 7 day embryonated eggs, and

further incubated for 9 days at 35°C. The harvested material was used for passage in two series. a. Amniotic fluid, and b. chorio-allantoic membrane and amniotic membrane ground up in their corresponding fluids. By back titrating in human amnion cell cultures, they demonstrated that the virus did in fact multiply, and they confirmed the identity of the virus by neutralisation tests.

c. Non-Primate Tissues

Dogs. In 1958 Frankel and King-West, successfully used dog kidney tissue culture to grow the measles virus. They were able to isolate 5 strains of measles virus by inoculating "clinical material" into the tissue culture tubes.

Further, they claim that these cells have the advantage that they are relatively free from extraneous viruses (e.g. foamy agents, MINIA etc. in monkey kidney) and have considerable longevity in cultures. The cytopathic effect seen was the formation of stellate as well as giant cells, with intra-nuclear and intra-cytoplasmic bodies and ultimate destruction of all cells.

d. Bovine tissues

Enders et al (1957) failed to grow the virus in bovine amnion tissue cultures, Warren and Cutchins (1957) met



with similar result when they used bovine kidney tissue cultures, but Schwarz and Zirzbel (1959) were more successful. They adapted the Edmonston strain, which had gone through 28 human kidney and 35 human amnion passages, to grow in bovine kidney tissue culture. In one set of tubes where no passages were made, and the maintenance fluid was only changed periodically, the typical cytopathogenic changes were seen after 70 days, and the virus was harvested up to 81 days. In another series where blind passages were made every 14 days, using both cells and tissue culture fluids, the cytopathogenic effect was seen after 8 passages. The identity of the virus was confirmed by serological tests.

e. Established cell-lines

Measles virus has been adapted to grow in several cell lines. Dekking and McCarthy (1956) adapted the Edmonston to grow in KB cell line, and obtained a cytopathic effect after 3 days. A syncytial formation was noticed and the cells soon dropped off the glass. In another 2 to 3 days these gaps were grown over and a fresh extensive syncytium appeared. The adaptation to HeLa and Hep -2 cell lines was more difficult. Virus grown in monkey kidney tissue cultures was used to inoculate HeLa cells by Black et al 1956. After 38 days incubation a non-specific degeneration was seen in

one of the tubes. When this material was further passaged to Hep -2 and HeLa cells, the HeLa cells again showed the non-specific degeneration, whereas giant-cells were seen in Hep-2 cells after 2 - 14 days depending on the size of the inoculum. Further passages from these HeLa tubes (7th passage material), showed the typical cytopathic effect. McCarthy and Rutigan (1956), and Wright (1957) also adapted the measles virus to grow in HeLa cells. Enders et al (1956) adapted the virus to the Detroit 6 cell line, as well as to a line of human embryonic fibroblasts, and Frankel and West (1953) used a human amnion cell line (W.S.). To summarise, the measles virus has been successfully grown in the following primary cultures:-

A. Human. Kidney.

Amnion.

Chorion.

Heart.

Skin fibroblasts.

B. Animal. Monkey kidney.

Dog kidney.

Bovine kidney.

Guinea pig kidney \*

Hamster kidney. \*

Mouse kidney. \*

Whole chick embryo and tissue culture.

C. Cell lines: HeLa. Human Heart Line: (Giradi).  
 KB.  
 Hep-2.  
 Human nasal (Jordan).  
 Human conjunctival (Chang).  
 Human kidney (Chang).  
 Rabbit E. R. K. (Westwood's embryonic  
 rabbit kidney) \*  
 W. S. Cells )  
 F. L. Line ) (Human amnion cell line)  
 (Meyer)

\* Cytopathic changes only. Viral multiplication not determined. (Modified from Viral infections of Infancy and Childhood. Page 219. Editor, Rose - Publishers: Hoeber and Harper).

### The Canine-Distemper Virus

#### Experimental Animals

Dogs, ferrets, foxes, mink, wolves, weasles, ermine and martens are all said to be susceptible to distemper virus. Perhaps the most susceptible of all these animals is the ferret and this is the experimental animal of choice. "It has long been the opinion of the keepers of ferrets that these animals are susceptible to canine distemper and that it occasions in them a highly infectious and fatal disease"

(Dunkin and Laidlaw 1926). According to these authors, Gray and Sewell succeeded in transmitting the disease to ferrets. The incubation period is about 10 days, after contact, and the first sign of infection is a watery nasal discharge, and reddening of the conjunctivae. A purulent conjunctivitis and nasal inflammation quickly follow. The chin becomes inflamed and vesicles appear around the lips. The feet swell and the abdominal skin may become red. On about the third day the vesicles become pustular and the animal weakens and usually dies on the 5th or 6th day with signs of pneumonia. Virus is present in the blood stream during the first three days and is found in large amounts in the spleen, consolidated lungs, and lymph glands as well as in other tissues. Pathologically the more important lesions are the inflamed mucous membrane which are covered by a purulent exudate, a bronchopneumonia with mucous plugs in the smaller bronchiolus, and an acute enteritis sometimes with ulceration of Peyer's patches. A frequent secondary invading organism is Bordetella bronchiseptica.

Green (1939) was able to infect ferrets with a strain of distemper virus, which he obtained from a sick fox and by passing it serially through the ferrets, i. e., a susceptible



but unrelated species, modified its virulence for foxes and dogs. This strain FXNO was developed and used as the "distemperoid vaccine". While not perfectly safe in the foxes and dogs it was nevertheless extensively used until the coming of the vaccines containing the less virulent egg adapted strain.

b. Rodents

It has been shown by Morse et al (1953) that suckling mice can be infected with the distemper virus. Using the FXNO egg-adapted distemper virus to inoculate 1 - 4 days old Rockefeller line of albino mice, they adapted two strains to suckling mice, and carried them over 10 and 18 passages. These authors have also isolated another strain by direct inoculation of the blood from a sick dog into suckling mice. (Morse 1956). Bindrich (1954) carried a strain of the virus through 14 mouse and 12 egg passages. Cabasso (1955), Gutiera and Gorman (1955), were able to adapt a strain not only to suckling mice but also to suckling hamsters. West and Brandly (1955) were able to infect mice with fox-distemper virus, by intranasal inoculation and carried it through 12 to 40 passages. Arakawa et al (1959) claimed that they too, had isolated three strains of distemper (hard-pad disease) directly in mice by inoculating the acute blood of infected dogs.

This is their "miniarni strain".

c. Chick embryos

Several attempts to grow the virus in the tissues of developing embryos were made. In 1938 Mitscherlich, as quoted by Cabasso and Cox (1949), was able to keep the virus alive on chorio-allantoic membrane for 6 days but could not transfer the infection. Plummer (1939) was a little more successful in that he was able to take the virus through 6 passages before losing it. Beveridge and Burnet (1946) failed to grow the virus in any of the chick tissue, though they used several routes. Two years later, Haig (1948) using Green's distemperoid strain, adapted the virus for the first time to grow on the chorio allantoic membrane, and maintained it for over 90 serial passages. He was unsuccessful when he used the allantoic, amniotic and yolk sac routes. He incubated his eggs at 37°C, and was able to observe definite macroscopic lesions. The virus, too, had lost some of its infectivity for the ferrets. Cabasso and Cox (1949) who had started their experiments in the same year as Haig (1948) were also able to adapt the virus to grow on the chorio-allantoic membrane, taking it once through yolk-sac as well. They used the "Lederle" strain which originally had been isolated in 1941 from a sick dog, and

which had been maintained ever since by "irregular alternate passages through ferrets and dogs".

This virus was fatal to ferrets and mildly pathogenic to dogs. They used the chorio-allantoic membranes of 7 day old eggs on which 0.2 ml. of a bacterially sterile supernatant from a 20% infected spleen emulsion had been inoculated. After 4 blind passages in eggs, using 20% chorio-allantoic membrane emulsion as the inoculum, the virus was found to be still present in the eggs. This was as demonstrated by the inoculation of ferrets, which succumbed without evident macroscopic lesions. The 20% spleen emulsions from these dead ferrets were taken through one yolk sac passage and three further chorio-allantoic membrane passages, and these were again tested in ferrets with fatal results. The spleen emulsion from their second set of ferrets was thereafter maintained by several passages (74 passages) on the chorio-allantoic membrane of eggs and tested in ferrets every 3rd or 4th passage. Macroscopic lesions were seen for the first time, in 9th and 10th passages. The membrane appeared swollen, moist, and had numerous minute greyish-white lesions, which became more numerous and more pronounced in the further passages.

The virus remained pathogenic to ferrets up to the 26th passage, after which it only retained its immunising capacity.

These authors confirmed the identity of the virus, by challenging the immunised animals, both ferrets and dogs with virulent strain of distemper virus and by neutralisation tests in eggs and in susceptible animals. The strain produced specific antibodies in both susceptible and non-susceptible animals.

d. Mammalian Tissue Culture

The next successful attempt at growing canine distemper virus was made by Dedie and Klapoke (1951, 1952) who used minced suspended tissues of dog spleen, lung, lymph nodes, and testicular tissues, in which the virus grew. They found, however, that it failed to grow in kidney explants. The presence of virus was detected by inoculation into susceptible dogs, because no discernible cytopathic changes were observed in the tissue cultures. These experiments were, however, inconclusive because the authors had no type strains with which to compare their results, nor did they use any neutralisation tests.

Rockborn (1958) failed in "several systematic attempts, spread over nearly two years" to grow the egg-adapted strain in tissue cultures, but they succeeded in culturing this virus, when the inoculum was changed from an adapted virus to a naturally occurring virus. Using tryptic



sinised dog kidney monolayers, they were able to grow the distemper virus, the inoculum being the blood from puppies in the acute phase of distemper. These animals had been previously inoculated with throat swab material taken from naturally infected dogs. A cytopathic effect was observed 42 days after the primary inoculation (Rockborn 1958)<sup>b</sup>, Hopper(1959)<sup>b</sup> and Vantsis (1959) using stained preparations were able to observe the cytopathic effects within 3 to 4 days when the inoculum (blood) was taken in the acute phase, but 4 to 5 weeks had to elapse before any change could be detected, when the blood was taken from the chronic cases. Rockborn found that after the 8th passage the cytopathic effect appeared in about 48 hours. The lesions started as scattered foci of swollen cells, many of which showed granular degeneration with vacuolation. Later, these fused to form a few multinucleate giant cells, which were characterised by a granular cytoplasm around which the nuclei were distributed peripherally amidst a broad band of vacuolated protoplasm. Nuclei could also be central but there is no mention of inclusion bodies. The identity of the virus was confirmed by inoculating the tissue culture fluid into susceptible and immunised ferrets and dogs and by neutralisation tests against known anti-distemper serum. Vantsis (1959)

used the kidneys and lungs from distemper infected ferrets and dogs to obtain trypsinised monolayer tissue cultures. He inoculated the clean tissue culture tubes with infective material from clinical cases. In all the tissue cultures obtained from both naturally infected animals and also in the inoculated tubes he was able to see the giant cells with peripheral phloxinophilic intracytoplasmic inclusion bodies and up to "a score or more of nucleii arranged in a circle". Intranuclear inclusion bodies were seen only in cultures kept over three weeks after inoculation. According to this author, these changes were seen in animal tissue cultures processed 6 to 7 days after infection, and 3 to 4 days after inoculation of healthy tissue culture monolayers. These findings do not agree with those of Rockborn, but Vantsis makes the statement that he was able to see these changes much more easily in the stained preparation he used than in the unstained cultures used by Rockborn.

e. Avian Tissue Cultures

Gillespie (1959) Cabasso et al (1959), and Karzon and Bussel (1960) claim to have been successful in growing the egg-adapted virus in chicken embryo fibroblast monolayer. Cabasso et al, however, were not able to observe any cytopathic changes in the tissue cultures of chick embryo fibro-

blasts and the presence of virus in the tissue culture fluid was detected by back inoculating into the eggs with virus grown in tissue culture fluid, when the definite and characteristic lesions were produced on the chorio-allantoic membrane.

Gillespie (1959) was able to observe the cytopathic changes when he used the medium made only with Earle's saline. Karzon and Bussel (1959), had similar results, and using a higher concentration of sodium bicarbonate (0.75 mg. per cent) were able to produce plaques as well. The affected cells were granular, swollen and rounded off. No giant cell formation was noticed. The titre of virus equalled that grown on the chorio-allantoic membrane, and according to Bussel (1960) the major part of the virus was cell associated.

Reynesh (1956) showed that the virus would pass through graded membrane filters with an average pore diameter of 210 mμ, but were retained by those of 190 mμ. This suggests that the diameter of the virus particle is approximately 140 mμ.

The measles virus is well preserved by freeze drying. Although there is some loss of infectivity after lyophilization the agent remains infective for at least 18 months after

## THE PHYSICAL PROPERTIES OF THE VIRUSES

### Measles

Although a great deal of information has been published on the properties of the measles virus, relatively little appears to have been recorded on the distemper viruses.

The following is a resumé of the known physical properties of the measles virus.

#### 1. Particle size

Goldberger and Anderson (1911) showed that the virus would pass through Berkefeld filters. In an electron microscopical study (measurements found in the literature) Reagan et al (1952) found that the measles virus, circulating in the blood of the infected monkey had a diameter of 90 - 100 mμ. No further measurements were made until Beynesh (1958) showed that the virus would pass through gradacol membrane filters with an average pore diameter of 210 mμ, but were retained by those of 190 mμ. This suggests that the diameter of the virus particle is approximately 140 mμ.

#### 2. Heat.

The measles virus is well preserved by freeze drying. Although there is some loss of infectivity after lyophilization the agent remains infective for at least 18 months after



storage, at  $+6^{\circ}\text{C}$ . (Musser and Underwood 1960). Prior to freeze-drying the virus is best suspended in a tissue culture medium to which 5% serum is added; this increases the stability of virus regardless of the temperature.

At  $37^{\circ}\text{C}$  and at room temperatures tissue culture fluids lose infectivity at the rate of approximately 1 - 2 log units per day. A table with the details (thermal inactivation of the measles virus) is given in the appendix.

### 3. pH levels

According to Black (1958, 1959) the measles virus is not affected by fluctuation between pH 5.5 and pH 9 <sup>at</sup> ~~and~~  $0^{\circ}\text{C}$  for three hours. There is an almost complete loss of infectivity below pH 4.4 (i. e. from 3.9 log units to 0.7 log units at pH 4.4) and relatively less loss at pH 10 (from 3.9 to 3.3 log unit).

### Photo-inactivation

As early as 1900, Rabb, reported the destructive action of light on micro-organisms. Skinner and Bradish (1954) drew attention to light "as a source of error in the estimation of infectivity of virus suspensions". Lozovskaia, in 1959, as quoted by Cutchins and Dayhuff (1962) reported the loss of infective-titre in suspension of measles and in influenza virus

which had been exposed to light during and after lyophilization. The above two authors, using measles virus grown in a human amnion cell line, showed that the rate of inactivation of the virus was directly proportional to the intensity of light, and the shorter the wave length, the quicker was the viricidal effect. Certain proteins, e.g. serum in concentrations of 10 to 20 per cent, had a protective action, as did the addition of some reducing agents like glutathione, and ascorbic acid, which helped in the oxidation process.

Other agents The virus is easily inactivated by ultra-violet light and exposure to ultrasonic vibration for two hours ( 10 KC/Sec) at 4.6°C completely destroys infectivity. (Girardiet al 1958)., but, according to Ackerman and Black (1961) this virus is more resistant to ultraviolet than to ionizing radiation.

The ultra-Violet ray irradiated virus grew well in normal monkey kidney cell culture, and its capacity to grow in ultra-violet ray irradiated monkey kidney cells was considerably reduced.

### Chemicals

#### 1. Formalin

Exposure for 5 days to 1:4000 formalin at 37°C com-

pletely destroys the infectivity of the measles virus, without, however, affecting the complement fixing property.

## 2. Glycerol

Resists 50% glycerol for 3 months. (Tarigandie et al (1935)).

## 3. Ether sensitivity

According to Rake et al (1959) the virus withstands 10 per cent ether at room temperature for 40 minutes, but Palmer and Black (1961) state that overnight treatment with 50% ether completely destroys the infectivity of the virus. Cooper (1961) has classified the virus as a "lipovirus" which is ether sensitive.

## Canine distemper

The virus passes through filters and, according to Palmer and Black (1961) is, by filtration experiments, 115 and 160 mu in diameter. An earlier electron microscopical measurement by Reagon and Brucknev gave the mean diameter of the virus particle as 25 mu. Tawara et al (1960) have described two kinds of inclusion-like bodies in the electron microscopic study of canine distemper infected urinary bladder of ferrets. One is a highly osmophilic dense body, the second type, less osmophilic and approximately 120 mu appears in the later stages.

## Heat

There is a considerable difference of opinion as to the stability of the virus to heat. It survives better in a fluid medium (Piercy 1962) or in the infected tissues such as lungs, spleen, lymph glands (Laidlaw and Dunkin 1926). The distemper virus appears to be, however, more resistant to thermal inactivation than the measles virus. Details of this thermal effect is given in the appendix.

Infective and Complement fixing antigens

Schluederberg and Roizman (1962), by ultra-centrifugation of measles virus concentrates in a cesium chloride density gradient, have been able to separate three measles antigenic fractions. The heaviest fraction, the pellet of which had a density of  $1.288 \text{ g/CM}^3$  had 99.6 per cent of the infectivity and only 2.7 per cent of complement fixing activity of the total. The other two fractions, with lower densities were non-infectious and contained almost all the complement fixing portion of the virus.

Recently Machlowitz et al (1961) by tryptic digestion of measles virus, have been able to sediment complement fixing antigen by ultra-centrifugation. There was considerable increase in the complement fixing titre, up to 185 per



cent, as compared with the undigested control preparations. However, this digested and sedimented antigen had lost all its immunizing and infective properties.

#### Haemagglutination and haemagglutination inhibition tests

Peries and Chang\* (1960), using red blood cells from several species of animals, found that only the red cells obtained from baboons were agglutinated by high titres of measles virus. This was seen best at 37°C, and was confirmed by Meio and Gover (1961). Rosen (1961) met with equal success using Rhesus monkey red blood cells. Using measles anti-serum he developed a haemagglutination inhibition test and demonstrated that this test was as sensitive and as specific as the complement fixation reaction, but with the added advantage of lacking the drawbacks associated with the complement fixation test. Rosanoff (1961) grew the virus in different tissue culture systems and found that the virus grown in primate tissue culture systems had both the haemagglutination and complement fixing properties; when grown in baboon kidney cells, the highest titres were obtained. Virus grown in chick embryo tissue culture, although highly infective, could neither fix the complement nor agglutinate Rhesus cells. This haemagglutinating property was comparatively stable and persisted for 3 to 4 days at 37°C.

Elution of the virus was not possible from the agglutinated cells. Tanned cells, laden with virus, did not give any higher titres of agglutination. Several species of animals immunised with measles antigens grown in heterogenous tissue culture systems, including that grown in chick fibroblasts, produced antisera with the Complement fixing and Haemagglutination inhibition titres. Specific human gamma globulin had both these antibodies as well.

#### Measles Interferon.

Ho and Enders (1959) had observed that tissue culture fluids, infected with measles virus, contained a substance that retarded both the growth of the virus as well as the cytopathic effect produced by it. Enders (1960) pointed out that avirulent strains of virus grown in human amnion cells gave a higher yield of this substance than was obtained with a virulent strain of the virus. The detection and the properties of this interfering agent were further elucidated by De Meyer and Enders (1961). They found that it was released at about the same time as the "infective virus", and its production increased in parallel with the cytopathic effect observed. When this substance was tested with polio and Sindbis viruses, there was no neutralization or inactivation of the viruses themselves, but their reproduction was

diminished. This interfering agent was entirely different from the infective and complement fixing viruses themselves, and could be easily separated by centrifugation for two hours at 30,000 R.P.M.; it was not neutralized by specific measles antiserum.

Again, in contrast to the infective virus, it was stable to heat 2 hours at 56°C and to ether 1 hour and 20% Ether by volume. The entire quantity passed easily through a 10-mpc millipac filter, where the infective particle was completely held back, as was most of the complement fixing particle. Ribo-nuclease and deoxy-ribonuclease did not affect this substance, but it was totally destroyed by trypsin, in contrast with complement fixing particle. This measles interferon is different from the influenzal interferon of Burke and Issaacs (1957) which is produced after the peak of virus production and is ether sensitive.

## THE RELATIONSHIP OF MEASLES AND

### DISTEMPER

#### The basis for an established affinity

Bryan (1928) first drew attention to canine distemper as a possible etiological agent of a human upper respiratory infection. He substantiated his hypothesis by both circumstantial and experimental evidence. Nicolle (1931) and Whitney (1943), basing their findings on clinical and pathological grounds were convinced of this possibility. Adams (1941, 1948), Adams et al. (1942) from their study of clinical epidemiological and serological findings laid emphasis on a relationship between canine distemper and a primary pneumonitis occurring in children. In 1945, Pinkerton et al. pointed to the striking similarity of the histopathological appearances seen in primary atypical pneumonia in children (Hecht's disease), canine distemper, and measles.

Subsequently several workers, Adams (1953), Imagawa et al. (1954), Karzon (1955), Rjazantseva (1956), Adams et al. (1955), Sergiev (1956), Carlstrom (1956, 57, 58, 59), Adams et al. (1957), Adams et al. (1958), Adams et al. (1959), Bech (1960), attempted to investigate this problem by means of serological and animal experiments. Others,



et al.

Warren (1960), Scharz (1960), Hoekenga (1960), used attenuated measles and distemper viruses for vaccination, in a common host species, and compared the antibodies produced.

On the other hand Cabbasso (1959), and his co-workers (1960) have tried to demonstrate the dissimilarity between the two viruses. They immunised ferrets with repeated injections of measles vaccine, and obtained very high levels of antibodies for measles virus. When challenged with a virulent strain of distemper virus, the animals were quite as susceptible as their controls.

### Clinical and Epidemiological

From what has already been written, it will be appreciated that the two diseases have several similarities. Both have an incubation period of almost the same duration i.e. about 10 days. The prominent premonitory signs of fever, coryza, conjunctivitis and upper respiratory involvement are strikingly similar in both. The "Rash", a constant diagnostic feature in measles, though described in distemper, is often evanescent. The frequent complication of distemper, viz; chorea (a demyelinating encephalitis) is seen less commonly in measles. Epidemiologically both these highly contagious diseases affect primarily the young population,

and after recovery, as a rule leave a life-long immunity. In a susceptible population both spread very rapidly.

#### Histopathological findings

It was known from the days of Hecht (1910), that in patients and monkeys who died during the pre-eruptive stage of measles, large syncytial, multinucleated, giant cells were seen in tissues such as the lungs (Alagena, 1911), lymph glands and spleen (Hathaway, 1935, Samsworth, 1939, Corbett 1945). Finkeldey (1931) and Warthin (1931), independently observed these giant cells in such lymphoid tissues as tonsils, adenoids and appendix, excised from patients during the incubation period of measles; they concluded that these "WARTHIN-FINKELDEY" bodies were pathognomonic for measles. McCallum (1939), Kreider (1943), however, failed to observe them in patients who died after the appearance of exanthemata.

Adams (1943) had postulated the giant cell pneumonia in children as possibly due to the canine distemper virus. This, however, was later disproved by the isolation of measles virus from these cases of Hecht's disease. (Enders et al. 1959) McCarthy et al. 1958).

In 1945, Pinkerton, Smiley and Anderson, pointed to the similarity of the lesions seen in the pulmonary lymphoid tissues of fatal cases of distemper in ferrets, minks and foxes, to that seen in children suffering from Hecht's disease. Adams et al. (1956, 1957) referred to the distinctive histopathological picture seen in various respiratory viral diseases, and noted that identical changes are seen in the pulmonary lesions in measles, interstitial pneumonia (Hecht) and in dogs and ferrets dying of distemper. A poliferative change was seen in the lining epithelium of the bronchioli, with giant cell formation and an infiltration of large mononuclear cells. Both cytoplasmic and intranuclear inclusion bodies were also present.

#### The Virological Evidence

In a comparison of the physical properties of the measles and distemper viruses Palmer and Black (1961) found that both were very similar in size. In filtration experiments through a gradacol <sup>membrane</sup> both were 115-160 mu in diameter. In Inactivation curves of both viruses after treatment with ultra violet light and gamma irradiation, little difference could be detected, and heat treatment at 37°C and 56°C appeared to affect the viruses equally. Both viruses



were either sensitive. These authors are of the opinion that their observations are compatible with the viruses being members of the same group.

### Serological evidence

Adams (1953) whilst investigating 2 cases of primary pneumonitis in children (one of whom was in contact with a dog suffering from distemper) demonstrated the presence of canine distemper virus neutralizing antibodies in the sera of the children. Later, he and his colleagues (1957) demonstrated by complement-fixation tests that the canine distemper antibodies could fix measles virus in titres up to 1:256. Imagawa et al. (1953, 1954) showed the presence of canine distemper neutralizing antibodies in normal prematurely born babies as well as in adult human sera - almost to the same level as that found in an immunised ferret. Karzon (1955) went further in the same field, to show that the heat stable neutralizing antibodies (to canine distemper) of the human mother were transferred to infants, <sup>and</sup> ~~that~~ persisted till they were about 6 months old and that they reappeared in adult life. Almost 100 per cent of adults had these antibodies. Monkeys had them as well. Carlstrom (1956) confirmed these findings, and found that children less than four years old



seldom had these antibodies for distemper whereas those above six invariably had them: this is related, as will be seen, to previous infection with the measles virus.

In an attempt to find out whether other infectious diseases were also responsible for the production of immune bodies to canine distemper, Carlstrom (1957) tested the paired sera obtained from children suffering from various other diseases. His findings were that there was no relationship to past infections with diseases such as mumps, chickenpox, rubella, exanthem subitum, and infectious mononucleosis. Only 17 out of 78 cases showed a low rise in antibodies, whereas 37 out of 38 cases of measles were positive for distemper antibodies. Using a mouse - adapted distemper strain, Carlstrom showed that there was a four-fold rise in canine distemper neutralising antibodies in the convalescent samples of human sera - titres varying from 1:5 to 1:625.

Hopper (1959), working in Great Britain estimated the levels of canine distemper virus antibodies in humans by age groups, and compared his findings with sera of children from other countries, viz. Nigeria, Iceland (where dogs are said to be rare) and Russia. His findings were that children of the age group 6 months to 2 years had no distemper

antibodies, from 2 to 6 years, there was a sharp rise, and the highest level was in the age group 18 to 20 years.

Hopper's work showed the presence of antibodies to canine distemper in all cases of measles with or without rashes.

Bech (1960) studied the rise and fall of complement fixing antibodies to both viruses, in five humans and two dogs. The antigens used were the distemper virus grown in dog kidney and the measles virus in dog and monkey kidney tissue cultures. Five measles patients' sera showed a high and rapid increase for measles antibodies (1:256 to 1:512); the highest titre was reached in 6 to 9 days after the rash; however, the highest titres of distemper antibodies (1:16) were a little delayed - 9 to 40 days after the rash. Bech tested the sera of the same patients at the end of 1st and 5th years. There was a very marked decline in the measles complement fixing antibodies (the results are summarised in tabular form) but the level of antibodies to distemper did not alter appreciably.

Table showing the levels of complement fixing (CF)  
antibody of measles (MV) and canine distemper (CDV)  
Antigens in the sera of five patients

Pts.No.	1		2		3		4		5	
Antigen	MV	CDV	MV	CDV	MV	CDV	MV	CDV	MV	CDV
Highest titre of CF anti-bodies in convalescent serum	512	16	512	16	256	16	256	8	256	4
At the end of 1st year	64	32	32	16	64	32	64	32	32	16
5th year	64	32	32	16	32	32	16	16	32	32

Thus Bech concluded that measles patients develop antibodies to the distemper virus to a titre 8 to 32 times lower than for the measles virus itself. During convalescence the titre of measles antibodies falls more rapidly so that there is a point at which antibodies to both viruses are equal in amount. In puppies, antibodies were developed for both viruses but to a higher titre for the homologous antigen.

#### Tissue culture and animal studies

Adams et al. (1957) have pointed out the identical nature

of the giant cells seen in tissue cultures, infected with measles virus (HEP - 2, HeLa and K. B. cells) and those seen in the pharyngeal and tracheal mucus membranes of ferrets experimentally infected with canine distemper virus. Cytopathic effects in the tissue cultures were neutralized by both human convalescent measles sera and specific canine distemper antisera. In ferrets immunised with measles vaccine, and later challenged with virulent distemper virus, they observed the incubation period was prolonged, and 8 out of 20 animals survived. The mouse adapted canine distemper virus was completely neutralised by specific measles antiserum, as well as by human convalescent measles serum - experiments that led these authors to say that there was a common antigenic component shared by these two viruses.

All these experiments were done with measles and distemper viruses, and their antisera derived from heterogenous sources; hence they were inconclusive. However, Carlstrom (1956, 1957, 1958)<sup>4.b.</sup> tried to overcome this objection by mouse-brain adapted strains of measles and distemper viruses. He used these two strains of viruses, as well as tissue culture grown measles virus to produce the respective antisera in rabbits. Thus, he had an animal species, equally



susceptible to both the viruses, and antisera for both, produced in the same species of animal:- a good basis for comparison. Carlstrom's findings were, a. the tissue culture grown and mouse adapted measles virus were antigenically identical, b. both measles and distemper viruses when adapted to mouse brain, produced an identical picture in mice, ultimately killing them.

In his neutralization experiments, he used an identical quantity of both antisera to neutralize, the minimum lethal dose of the two viruses, i.e., a constant quantity of virus and variable serum. He found that the canine distemper virus was neutralized by both anti-distemper and anti-measles rabbit sera, to the same extent, whereas, with measles virus the heterologous sera had a much lower neutralizing titre than the homologous sera, the ratio of the titres being 1/25. Hence he concluded that there existed an antigenic relationship between the two viruses.

Mention must be made of two interesting papers by the Russian workers - Ryantseva<sup>2a</sup> (1956) infected 48 puppies with measles virus, of various origins viz. throat washings from measles patients, the viraemic blood of a rabbit dried at 20°C, using the conjunctival, intranasal, pharyngeal (by

rubbing), and subcutaneous routes. He found that some of the puppies suffered from a mild disease; about 50 per cent of dogs showed a pyrexia and 38 per cent a rash.

Sergiev (1956) also infected 13 puppies, and the puppy-passaged measles virus used as vaccine, protected susceptible monkeys, when challenged with virulent measles virus. However, as full details are lacking, the interpretation of these findings is difficult.

In order to study this problem further a survey was

made of the properties of the two infective agents. Obser-

vations were made on the properties of the viruses to include

their morphology under the electron microscope, their

chemical nature and content, some of their

physical properties, their pathogenicity in a range of labora-

tory animals, their host range and growth characters in a

number of systems of tissue culture and eggs, and their anti-

genic structure and potency. In the course of these studies it

was hoped that it could be decided whether the two viruses

belong with the virus of rinderpest a common group, whether

infection in species other than their natural host resulted in

attenuated disease and if the latter were true, whether the

From the preceding pages it can be seen that there are many striking resemblances between measles and distemper; in their pathogenesis, clinical features and in their epidemiology they are so similar that it might be wondered whether they are caused by a single infective agent. Yet measles is a human disease and distemper a canine one. There is no proof that measles can be contracted from a dog with distemper and neither is it known whether human measles can be transferred to the dog.

In order to study this problem further a survey was made of the properties of the two infective agents. Observations were made on the properties of the viruses to include their morphology under the electron microscope, their chemical nature and nucleic acid content, some of their physical properties, their pathogenicity in a range of laboratory animals, their host range and growth characters in a number of systems of tissue culture and eggs, and their antigenic structure and potency. In the course of these studies it was hoped that it could be decided whether the two viruses form with the virus of rinderpest a common group, whether infection in species other than their natural host resulted in attenuated disease and if the latter were true, whether the

resulting degree of cross immunity would be of protective value.

The foregoing survey of the literature has made it abundantly clear that no completely satisfactory comparison of the viruses of measles and distemper has yet been made. Two main difficulties have been encountered; first, there has been no common susceptible host in which to study the two natural infections; and second, it has been scarcely possible to find any suitable system in which both the viruses can be propagated under identical natural conditions. Furthermore, the fact that both diseases are widespread in nature from an early age has destroyed any value that might be obtained from a serological analysis of antibody levels in human or canine populations. Lastly, the degree of mutability of the two viruses has not been assessed although it is assumed to be great in distemper.

In planning the work of this thesis the following objectives were set:-

1. The isolation of wild virus from patients suffering from a first and natural attack of measles and distemper.
2. The estimation of antibody levels arising under those circumstances.
3. The preparation of immune antisera by the infection of



experimental animals with the two viruses.

4. The preparation of hyper-immune sera by parenteral inoculation of rabbits, guinea pigs and ferrets.
5. The comparison of the properties of these sera in neutralisation, complement fixation, animal protection tests, and their examination by new immulogical techniques.
6. A comparison of the growth characters of the two viruses in a single tissue culture system.
7. A study of the external morphology, size and internal structure of the two viruses by electron microscopy.
8. The determination of the type of nucleic acid contained within the virus by fluorescent staining techniques and also by studying the growth inhibitory effect of amino-acid analogues of nucleic acid synthesis.
9. A re-examination of the pathogenesis of the infections in various laboratory animals with special reference to histopathology, inclusion body formation and changes within the host cell during virus growth.

## MATERIALS AND METHODS

### VIRUS STRAINS

The following virus strains were used in this study.

#### Measles

The Edmonston strain which was originally isolated in the United States of America by Dr. John F. Enders,

Two lines of this virus were used.

- I. A strain adapted to grow in human amnion (Strain No. 293/14, 16, 17) was kindly supplied by Dr. R. McCarthy of the University of Liverpool.

It was propagated only in primary human amnion tissue cultured during this study.

### METHODS & MATERIALS

- II. A strain adapted to grow in the HEP-2 line of cells was obtained through the courtesy of Miss G. D. Lawrence of the Wellcome Research Laboratories.

The "Berlin" strain. This was isolated from the blood of a patient in the acute phase of measles. It was obtained by inoculating whole blood at the bedside into monolayers of monkey kidney cells and was later adapted to grow in primary tissue cultures of human amniotic membranes.

#### Distemper

- I. The "C.D. 9" strain of canine distemper virus was

## MATERIALS AND METHODS

### VIRUS STRAINS

The following virus strains were used in this study.

#### Measles

The Edmonston strain which was originally isolated in the United States of America by Dr. John F. Enders.

Two lines of this virus were used.

i. A strain adapted to grow in human amnion (Strain No. 283/14, 26.29) was kindly supplied by Dr. K. McCarthy of the University of Liverpool.

It was propagated only in primary human amnion tissue cultures throughout this study.

ii. A strain adapted to grow in the HEp-2 line of cells was obtained through the courtesy of Miss G. D. Lawrence of the Wellcome Research Laboratories.

iii. The "Allan" strain. This was isolated from the blood of a patient in the acute phase of measles. It was obtained by inoculating whole blood at the bedside into monolayers of monkey kidney cells and was later adapted to grow in primary tissue cultures of human amniotic membranes.

#### Distemper

i. The "C.D. 9" strain of canine distemper virus was

received in the form of ferret spleen, suspension freeze-dried in ampoules, and made up in 10 per cent serum broth for use.

ii. The "Epi~~v~~ax" strain is a commercial product and has been attenuated and adapted to the chick embryo. It was propagated on the chorio-allantoic membrane of 6 - 7 day chick embryos.

Both these strains were supplied by Dr. S. P. Piercy of the Wellcome Research Laboratories.

iii. "Wild Strain I" was isolated locally from the blood of a dog in the acute phase of distemper. A ferret which received 2 ml. of whole blood subcutaneously died 15 days later with typical lesions. An uninoculated cage mate of this animal contracted the infection and was killed on the 24th day when it was moribund. A 20 per cent <sup>splenic</sup> suspension from the second animal was used in further passages.

iv. "Wild Strain II". Whole blood was drawn from the second <sup>ferret</sup> ~~animal~~ in the above experiment on the third day of fever and was inoculated into monolayers of dog kidney tissue cultures. A cytopathic effect was seen on the 14th day. The fluid from these tissue cultures



was used for further passages in ferret kidney tissue cultures; that it did in fact contain the virus was proved when it was given intranasally to a ferret which developed typical distemper 19 days later.

v. Wild strain III Three seven-week old puppies diagnosed as suffering from alimentary type of distemper, were anaesthetised and exsanguinated. Blood was collected in heparin (20 mg/per cent) centrifuged and white cell layer harvested, as described before for measles. This harvest was then inoculated intranasally into a 8 week old ferret, which later developed distemper. The clinical chart of this ferret and its cage mate, along with that of a third ferret, which was inoculated with the Virulent "C.D. 9" strain is given later (page 58 ).

Vaccinia virus A strain derived from the Lister Institute lapinised seed virus and propagated in Monkey Kidney tissue cultures.

Polio virus Type I. A reference strain which originally was supplied by Dr. Macrae of the Central Reference

Laboratory at Colindale.

## ANTISERA

### Measles

- i. Paired acute and convalescent sera were collected from patients with measles in the City Fever Hospital. Samples of convalescent sera were also used. Only those cases which were regarded by the physician in charge as typical measles were studied.
- ii. As a reference, a rabbit anti-measles serum was used. It was supplied by Miss G.D. Lawrence of the Wellcome Research Laboratories.
- iii. Rabbit anti-measles sera were prepared as follows:-  
The antigen comprised undiluted tissue culture fluid harvested from amniotic cell cultures when 75 per cent of the cells were showing viral damage. Its infective titre was  $10^{3.5}$  TCID<sub>50</sub>/ml. Before inoculation cell debris was removed by centrifugation for ten minutes at 2000 r.p.m. Two rabbits received 1.0 ml. of this antigen subcutaneously and a second dose of 1.0 ml. was given two weeks later by the same route. A third inoculation of 1 ml. was given intra-peritoneally two weeks after the second dose. The animals were bled

by cardiac puncture before and after immunisation.

iv. Ferret anti-measles serum was prepared by the above technique.

v. Guinea pig anti-measles serum was prepared from six animals using the same method. Serum obtained from the blood of these animals was separated and stored at  $-30^{\circ}\text{C}$  without the addition of preservatives.

(b) Distemper

i. Blood was obtained from 5 dogs which were either convalescent from or in the chronic stage of naturally acquired distemper.

ii. As a reference a horse anti-distemper antiserum was used. It was supplied by Dr. S.E. Piercy of the Wellcome Research Laboratories.

iii. Rabbit anti-distemper sera were made using the same inoculation schedule as for measles. In this case the antigen used for the first two inoculations was the avianised virus, the "Epi~~pi~~ax" vaccine. The third (intraperitoneal) injection consisted of the "C.D. 9" virus.

iv. Ferret anti-distemper sera were made by the following method.

Two male ferrets were inoculated subcutaneously with 1 ml. of the avianised "EPIDAX" vaccine and four weeks later were challenged with an inoculum of 1 ml. of a freeze-dried ferret spleen emulsion of the "C. D. 9" strain of distemper virus given subcutaneously.

Two weeks later a further infection of 1ml. of "C. D. 9" was given intraperitoneally.

The animals were bled by cardiac puncture before inoculation and 10 days after the third inoculation.



## MEDIA. AND SALT SOLUTIONS

The following solutions were employed in tissue culture procedures:-

### Balanced Salt Solutions

(a) Hanks' solution.

(b) Earle's solution.

(a) and (b) were made up according to the instructions in Mackie and McCartney's handbook of Bacteriology (10th Ed. 1960).

(c) Phosphate buffered saline (P.B.S.) was made as follows.

(Dulbecco and Vogt 1954).

Solution (a)	NaCl	8.0 g.
	K Cl	0.2 g.
	Na <sub>2</sub> HPO <sub>4</sub>	(anhydrous) 1.15 g.
	KH <sub>2</sub> PO <sub>4</sub>	(anhydrous) 0.2 g.
	Ion free distilled water	800 ml.

Solution (b)	CaCl <sub>2</sub>	(anhydrous) 0.1 g.
	Ion free water	100 ml.

Solution (c)	MgCl <sub>2</sub> , 6 H <sub>2</sub> )	0.1 g.
	Ion free water.	100 ml.

Solutions A, B and C were autoclaved separately cooled and mixed together. Care was taken to ensure that no precipitate formed.

(d) Sodium bicarbonate - 1.4 per cent and 4.4 per cent in Ion free water.

### Defined Media.

Medium 199 (~~Parker and Healy 1955~~) obtained commercially from Glaxo Laboratories.

Eagle's medium (modified by Paul 1960) obtained commercially from Messrs. Burroughs Wellcome.

### Natural Media.

Lactalbumen hydrolysate was dissolved in Hank's or Earle's solution and used at a concentration of 0.5 per cent.

Tryptose phosphate broth (Difco) 3 per cent, dried powder in water.

### Sera

Horse serum Obtained commercially from Evans Medical Limited.

Calf serum Obtained locally from 1 - 2 day old calves at the slaughter house. After separation from the clotted blood the serum was Seitz filtered and inactivated for 30 minutes at 56°C

Bovine Amniotic Fluid obtained from the gravid uteri of cows at the slaughter-house and treated by the method described by Mackie and McCartney (1960).

Both the sera and the amniotic fluid were tested for cytotoxic effects in tissue culture before use.

Antibiotic mixture for addition to culture media and tissue-

suspensions.

Sodium penicillin	1,000,000 units.
Streptomycin hydrochloride	500,000 ug.
Sterile P.B.S.	100 ml.

1 ml. of the mixture was added to 100 ml. of the medium.

Mycostatin (Squib) A stock suspension P.B.S. was prepared to give a concentration of 5,000 units per ml. For use 1 ml. of stock solution was added to 100 ml. of the medium to give a concentration of 50 units per ml.

This was not used as a routine, but only when there was an indication.

Chloromycetin, (Parke Davis) was used in a concentration of 20 mg. per ml., a suspension in solutions used for collection and processing of placenta, and in the growth media, until the monolayer formed was ready for inoculation. This was used in lieu of the antibiotic mixture mentioned above, and controlled the contamination better. However, when used with quick growing cells like HeLa and H. E. p. 2 it was found to be toxic.

#### Enzymatic and chelating agents

Trypsin and Pancreatin (Trypsin and Pancreatin solutions):

(Modified from Ayerback and Grobstein, 1958) 1.5 per cent stock solutions were prepared as follows:-

10 gms. of Trypsin (Difco 1:250) and 5 gms. of Pancreatin (Difco U.S.P.) were made into a thick paste with about 200 ml. of P.B.S. Then the volume was made up to 1,000 ml. This mixture was first coarse filtered on Whatman No. 1 filter paper, and then Seitz filtered. After testing for sterility and checking the pH to 7.4 (if necessary by the addition of sterile 1.4 per cent sodium bicarbonate solution), was stored at  $-30^{\circ}\text{C}$  in 20 ml. amounts.

For use, 1 Part of this stock was diluted with 4 or 9 volumes of P.B.S. to give the required concentration of 0.3 per cent or 0.15 per cent solutions.

#### Trypsin solution:-

Prepared and used in the same way except that pancreatin was omitted, instead 1.5 gms. of trypsin was used. This was diluted only with 4 volumes of P.B.S. to give 0.3 per cent solution.

#### Versene solution

Stock solution: 0.5 gms. in 100 ml. of Calcium Magnesium free P.B.S. (Dulbecco A. Paul 1960). This gave a ten times concentrated solution, without glucose.

For use:-      Stock solution    100 ml.  
                   Ion free water    900 ml.  
                   Glucose                2 gms.

Autoclaved at 10 lbs. for 10 minutes in 100 ml. quantities.



Dye SolutionsMETHODS

## A. Methyl Violet (Light &amp; Co) solution.

0.1 gm. of methyl violet dissolved in 0.1 M  
citric acid solution.

## B. Crystal Violet (Light &amp; Co) solution.

1 gm. dissolved in 100 ml. of P. B. S.

a. Human embryonic kidney from an embryo kindly  
supplied by Professor Macdonald of Aberdeen  
University.

c. Human lung. Biopsy specimen received from the  
The Operating Theatre in City Fever Hospital.

d. Rabbit kidney - by courtesy of Physiology Department,  
University of Edinburgh.

e. Dog kidney and lungs - by courtesy of Royal Dick  
Veterinary College.

f. Rabbit kidney and lungs.

g. Calf kidney.

h. Sheep kidney.

i. Rabbit kidney.

Secondary cultures

From monkey kidney monolayers, received from  
Messrs. Glaxo Laboratories and from the M. R. C. laboratory at Mill Hill. Because the "foamy agents" interfered  
with maintenance of strains, the cells received from Glaxo  
were discontinued.

Cell lines

REP - 2, was kindly supplied by Dr. Groll at Newell

## METHODS

The following tissues or organs were processed for tissue culture:-

### Primary monolayers

- a. Human amnion.
- b. Human embryonic kidney from an embryo kindly supplied by Professor MacDonald of Aberdeen University.
- c. Human lung. Biopsy specimen received from the the Operating Theatre in City Fever Hospital.
- d. Baboon kidney - by courtesy of Physiology Department, University of Edinburgh.
- e. Dog kidney and lungs - by courtesy of Royal Dick Veterinary College.
- f. Ferret kidney and lungs.
- g. Calf kidney.
- h. Sheep kidney.
- i. Rabbit kidney.

### Secondary cultures

From monkey kidney monolayers, received from Messrs. Glaxo Laboratories and from the M.R.C. laboratory at Mill Hill. Because the "foamy agents" interfered with maintenance of strains, the cells received from Glaxo were discontinued.

### Cell lines

HEP - 2, was kindly supplied by Dr. Grist of Ruchill

Hospital, Glasgow, and by Miss G.D. Lawrence of Wellcome Research Laboratories. HeLa - maintained in this laboratory.

#### Human Embryonic Lung

A rapidly growing line of human embryonic lung tissue in its eleventh passage after primary cultivation was obtained from Dr. Sonnabend of the City Wellcome Laboratory at the City Fever Hospital. It is probable but not yet determined that this was a "transformed cell line".

### TISSUE CULTURES

#### A. HUMAN AMNIOTIC MEMBRANE

Several methods for processing the amniotic membrane have been described; all are modifications of the original method first described by Zitcer et al (1955). The variations have been chiefly in the method of collection of the placenta, time of trypsinization, the concentration of the trypsin solution employed, pre- and post- trypsinization treatment of the membranes and cells, and in the composition of the growth-medium employed. A table is appended to show the important variations in the different methods employed by various authors.

The following technique was finally adopted.

#### Collection of the placenta

Table to show the various methods used for processing human  
amniotic membrane

AUTHORS	Collection Storage (room temp)	Stripping, cutting, washing.	Trypsinisation (a)	Washing Cell count	Growth Medium
ZITCER et al. (1955)	P. B. S.	2 mm. pieces	20' fractions	One wash in Hanks' BSS.	199 + 20% Ox serum
BECKER et al. (1957)	Sterile towels None.	3 to 4 large pieces in P. B. S.	0.1% overnight	P. B. S. <u>800 RPM</u> 8 min	Hanks' BSS + 0.5% LAH + 20% calf serum.
OSTERHOUT et al. (1959)	Hanks' SS. up to 2 hours.	P. B. S. 2 cm <sup>2</sup>	6 hours.	P. B. S. <u>2000 RPM</u> 15 min	Eagles + 15% horse serum.
PUCK et al.	Hanks' BSS.	Mince to fine (1 mm) fragments and washed twice in Hanks' less Ca. Mg.	0.05% in Hanks less Ca. Mg. 15-45 mins. / 38°C.	No washing	Direct dilution with 9 vol. of growth medium and incubated at 38°C in 5% CO <sub>2</sub> No preliminary trypsinization.
FERGUSON and TOBIN. (1958)	Immediate.	Membrane stored in 199 + serum overnight	3 to 5 hours	P. B. S.	199 + 20% human serum.
RUCKLE (1957)	Sterile pan	P. B. S. with extra anti- biotics.	30' fraction.	Hanks' BSS	Hanks' BSS + 0.5% LAH + 20% calf serum
PARKER (1961)	Hanks' S. S. up to 10 hours	Entire membrane wash in Hanks' BSS.	1 - 2 hours.	P. B. S. <u>1000 RPM</u> 20 min.	Hanks' BSS. + 0.5% LAH + 20% human serum.
DUNCAN & BELL (1961)	Hanks' SS. up to 9 hours	Wash in P. B. S. up to 6 pieces.	4 hours	P. B. S. <u>1000 RPM</u> 10 min.	Hanks' BSS. + 0.5% LAH + 20% human serum + 0.1% Bovine Albumin.

(a) TRYPSINISATION.

All trypsinisations carried out at 37°C with 0.25% trypsin solution in P. B. S. or Hanks' BSS, pH adjusted to 7.4 or 7.6; the first fraction is discarded, in all cases, unless otherwise stated.



Entire placentae obtained from a "Caesarian-section" were preferred. Those, however, from normal deliveries were also processed. The labour-ward nursing staff were specially requested to see that no disinfectants came in contact with any part of the placenta. To obviate this, the staff were requested to receive the placenta into a large piece of sterile gauze or a towel, to wrap it up and then to transfer it to the sterile 4-litre beaker supplied. The beaker contained 4 to 500 ml. of unbalanced Hank's salt solution, fortified with antibiotics in the following proportions:-

500 units of Penicillin,  
500 mg. of Streptomycin,  
and 50 units of Mycostatin / per ml.

The beaker was covered with two layers of sterile aluminium foil.

The placenta reached the laboratory within a half-hour of delivery, and the amniotic membrane was stripped immediately. The umbilical cord was clamped at its root with a sterile 7" Spencer-Wells artery forceps, and suspended from a clamp. The amniotic membrane was picked up at a point about an inch away at the root of the cord and a nick made with Mayo's scissors. The closed point of the scissors was then introduced into the nick made, then opened

out, so that the filmy amnion was separated from the underlying chorion, and the amnion was cut in a circle one to one and a half inches away from the cord, thus avoiding the concentration of mucins and hyaluronic acid (Parker 1961). The cut edge of the amnion was gripped with forceps and stripped down into about 10 large pieces. Any large or obvious blood clots were picked out or scraped off. These amnion pieces were transferred to 250 ml. sterile beakers containing 100 to 150 ml. phosphate buffered saline with the antibiotics, and allowed to remain for 15 to 30 minutes. Meanwhile, small portions from different parts of the membrane were removed, spread on a slide and examined microscopically. If the cells looked clean and good, i. e. cells with clear-cut outlines and finely granular cytoplasm, they were processed further. If the membranes looked unhealthy, ie. indistinct outlines with coarsely granular cytoplasm, the membranes were discarded (Parker 1961). This examination of the membranes was being carried out from the beginning of 1962. If the membrane was grossly lacerated, oedematous, or coloured it was discarded.

Washing of the membranes. The pieces were well swirled and rinsed in the phosphate buffered saline with antibiotics and rinsed in the phosphate buffered saline with antibiotics and

temperature (15 - 22°C). For this purpose the trypan-

then transferred to another beaker, piece by piece. This process was repeated five to six times, after which the pieces of membrane were free of all blood clots, appeared clean, and were of a delicate pink colour.

Trypsinisation was carried out either the same day or overnight. 0.3 per cent Trypsin-pancreatin mixture in phosphate buffered saline or in Balanced Hanks' solution was used. Antibiotics were added.

The membranes were then transferred to 250 ml. wide-mouthed glass stoppered bottles containing 100 ml. of the above Trypsin-pancreatin mixture, and maintained 30 minutes at 37°C. After shaking at 10 minute intervals, the resultant turbid fluid was discarded.

Method A. (Same day processing)

The membranes were then transferred to a Roux bottle, containing about 150 ml. of the above pancreatin-Trypsin mixture, tightly corked, and incubated for a further three hours at 37°C with occasional shaking of the bottle.

Method B. (Overnight Trypsination).

The membranes were transferred to a Roux bottle, containing 150 ml. of 0.15 per cent Trypsin-pancreatin mixture, trypsination was continued overnight at room temperature (18 - 20°C). For this purpose the trypsin-

pancreatin mixture was diluted with an equal amount phosphate-buffer-saline so that the diluted fluid contained 0.1 per cent Trypsin and 0.05 per cent pancreatin.

### Harvesting

Further processing was common to both the long and short trypsinization methods.

From these Roux bottles, the turbid fluid was carefully pipetted out to a sterile 250 ml. quantities <sup>bottle. 100 ml of P.B.S.</sup> ~~were~~ poured on to the membranes, and well shaken for two to three minutes after which the fluid was harvested. This was repeated once more, and both washings pooled. The first fraction and the washings were centrifuged at 1000 R.P.M. for 10 minutes, and the supernatant was discarded. The sediments were taken up in 50 ml. of P.B.S. with antibiotics, well mixed, and pooled and spun again as before. The washing was repeated once more. The sediment thus obtained was added to 20 to 50 ml. of growth medium (minus the serum) and well mixed with a pipette.

Cell counts, both viable and non-viable, were made. For viable counts, one part of cell suspension and one part of 0.1 per cent methyl violet in 0.1 M citric acid solution, were well mixed and the cell counts made in haemocytometer.



If the cell count was very high then, to one part of cell suspension, 2 or 3 parts of the dye were used. For nonviable cell count 1 per cent trypan blue was used (Becker et al 1958). If the ratio of the viable to non-viable cells was 9 to 1 or higher, the cells were diluted to give 300,000 to 5000,000 cells per ml. and dispensed into tubes and bottles. If the proportion of non-viable cells was greater, the cell-suspension was diluted to give a greater concentration of cells, but even then growth of the monolayer was usually unsatisfactory.

The cells were dispensed in tubes the same day, or were stored overnight at room temperature in complete growth medium (proportion - one volume of packed cells to 19 volumes of growth medium).

For 6" x  $\frac{1}{2}$ " tubes 0.5 ml cell suspension was used and for 5" x  $\frac{5}{8}$ " tubes 0.75 - 1.0 ml. The tubes were kept stationary in racks at 36°C for the next 24 hours, after which they were drained of fluid and fresh growth medium was added. The tubes were then placed in a rotating drum and a monolayer formed in 5-12 days. The cells were maintained by weekly changes of maintenance medium.

### Growth Medium

0.5 per cent lactalbumen hydrolysate	
in Hank's solution	61 ml.
3 per cent tryptose phosphate broth	15 ml.
Horse or calf serum	20 ml.
Antibiotics	1 ml.
1.4 per cent sodium bicarbonate	3 ml.

### Maintenance Media

1. 0.5 per cent lactalbumen hydrolysate  
    in Hank's solution 78 ml.  
    3 per cent tryptose phosphate broth 15 ml.  
    Horse or calf serum 3 ml.  
    Antibiotics 1 ml.  
    1.4 per cent sodium bicarbonate 3 ml.
2. Medium 199 (Parker) 97 ml.  
    Horse or calf serum 3 ml.
- 3 0.5 per cent lactalbumen hydrolysate  
    in Earle's solution 90 ml.  
    4.4 per cent sodium bicarbonate 5 ml.  
    Horse or calf serum 3 ml.  
    Antibiotics 1 ml.

Using media 1 or 2, cells could be maintained in viable form for as long as three months. Medium 3 was preferred for use after the cells had been inoculated with viruses because the cytopathic effect was markedly enhanced.

### B. SOLID ORGANS

The kidneys of various species of animals, lung tissue and human embryonic tissue were treated by a modification of Younger's<sup>n</sup> method (1954). As an example the procedure for dispersal cultures of ferret kidney cells is given below

in detail.

Young ferrets 2 - 8 weeks old were anaesthetised by the deep intra-muscular injection of 1 ml. per 2.5 lb. body weight "Veterinary Nembutal" (Phenobarbitone sodium: Abbott Laboratories) 60 mgm per ml., and were then bled out by cardiac puncture.

With sterile precautions the kidneys were removed whole with the perinephric fat and were dropped in sterile P.B.S. with added antibiotics. After thorough washing they were stripped of their fat and decapsulated. The kidney was then gripped at the hilum with forceps and the cortex was torn away in 2 - 3 mm. fragments until only the calix and the medullary area remained. The cortical fragments were placed in 25 ml. P.B.S. and were then washed until the supernatant fluid was clear. After the final wash the P.B.S. was replaced with warm 0.2 per cent trypsin in Hank's solution which had been adjusted to pH 7.6 by the addition of 1.4 per cent sodium bicarbonate solution.

A sterile teflon covered magnet was introduced into the flask and the suspension was slowly stirred for 15 minutes at 37°C. The supernatant fluid from this first trypsinisation was discarded and 20 ml. of fresh warm trypsin solution was

added to the drained kidney fragments. At 10 minute intervals on four or five occasions, the trypsin solution with the freed kidney cells was removed and replaced. The fractions so obtained were centrifuged and the supernatant fluid was discarded. The deposited cells were then resuspended in 10 ml. of the Hank's growth medium (less serum) as described above, and counted in the haemocytometer chamber. This suspension was then further diluted with the complete growth medium to give a concentration of 250,000 cells per ml., and was then dispensed into tubes which were corked and incubated at 36°C in stationary racks for 48 hours. On the third day the growth medium was changed and again on the six or seventh day.

Often cultures were made in 4 oz. medical flat bottles as well as in the tubes described above. For each bottle 10 ml. of the final cell-suspension was used and a good monolayer formed usually within seven days. Such cultures in bottles were used in three ways, (a) for immediate cultivation of viruses, (b) for plaque counting methods, and (c) for the preparation of secondary cell cultures. For the last named purpose the cells were stripped from the side of the bottle by exposure to the trypsin solution after 8 - 10 minutes at 37°C - as short an exposure as possible was used - and as soon as the cell sheet was seen to be partly detached the



process was terminated by washing the sheet free and dispersing it by active pipetting. The detached cells were then washed, counted and resuspended in growth medium to give concentration of 75 - 100,000 per ml. This suspension was then dispensed into tubes and uniform monolayers were obtained 4 - 5 days later.

This method was used successfully for dog, sheep and rabbit kidney and, on one rare occasion, for baboon kidney. It was found possible to store the cortical fragments of kidney overnight in P.B.S. with 1 per cent glucose and antibiotics + 4°C.

#### Lung tissue (Human).

Excised portions of lung were finely minced with scissors and it was necessary to ensure that smaller fragments (1 - 2 mm. cubes) were produced. Prolonged rinsing and washing was needed both before and after mincing; usually the fragments were rinsed 7 - 8 times. Thereafter the tissue was trypsinised in exactly the same manner as that described above.

## STAINING PROCEDURES

Cells grown routinely in roller-tubes, or in cover-slips introduced into stationary tubes, or in Leighton tubes, were used for staining.

The preparations when deemed fit for staining, were first washed in warm (37°C) phosphate buffered saline twice and were then fixed with one of the following fixatives: (Mackie & McCartney, 1960)

Formal saline,  
Methyl alcohol,  
Corrosive sublimate fixative,  
Ether alcohol mixture, or -  
Carnoy's fixative.

Control preparations of normal uninfected cells were prepared and stained in exactly the same way.

### 1. Giemsa Staining

Monolayers were fixed, stained and stripped with collodion and mounted on glass slides, by a modification of the method described by Pannel and Cordle (1960).

Coverslips preparations after fixation, with methyl alcohol, were stained with giemsa (1:20 dilution with buffer at pH. 7) overnight. Differentiation was with acetone or absolute alcohol.

### 2. Haematoxylin and Eosin staining

The staining procedure adopted was the one described

in Diagnostic Procedures (1956). 5 per cent acetic acid was added to the Harris haematoxylin, and 0.5 per cent alcoholic solution of Eosin was used.

### 3. Phloxine-Tartrazine staining

Coverslip preparations were washed in warm saline, fixed in corrosive sublimate, and stained by haemalum-phloxine-tartrazine method after mordanting in potassium dichromate (Penny's modification of Lendrum's method 1947).

### 4. Acridine orange (Fluorochrome) staining

Both coverslip and roller-tube preparations were used; these preparations were washed in saline and fixed either in corrosive sublimate fixative or in Ether alcohol mixture.

After fixation, the preparations were hydrated in graded alcohols of 80 per cent, 70 per cent and 50 per cent, then washed twice in citric acid-phosphate buffer (pH 3.8) and stained with 0.01 per cent acridine orange solution made in the same buffer. The excess of stain was washed off with the buffer.

The stained coverslips were mounted wet and examined with a Watson "Service" microscope. The illuminating source was a high pressure mercury vapour lamp whose rays passed through a Kodak Wratten 18A filter. A yellow green filter (Wratten 2B) was incorporated in the eye piece.

## ELECTRON MICROSCOPY

Throughout this work an E. M. 6 Associated Electrical Industries electron microscope was used with an accelerating current of 50 Kilovolts. Specimens were mounted on copper grids which had been covered with a collodion membrane and coated with vaporised carbon.

### Preparation of Viruses

#### Measles

##### (a) Shadowed preparations

The virus from 160 ml. of tissue culture fluid obtained from monkey kidney cells was purified by cycles of differential centrifugation. This starting material had an infectivity titre of  $10^{-3.5}$  in tissue cultures.

The first step was to clarify the fluids by centrifugation for 20 minutes at 2 - 4°C in a Spinco Model L centrifuge using an angle rotor No. 21 at 6,250 r.p.m. (3,000 G). The supernatant was then subjected to centrifugation for 90 minutes in a No. 40 angle rotor at 38,000 r.p.m. (127,000 G). The deposits thus obtained were resuspended in distilled water to a volume of about 12 ml., and this was given a clarifying spin for 15 minutes at 4,000 r.p.m.

The supernatant from this step was transferred to two tubes and placed in a swing-out rotor No. 39, and again sub-



jected to centrifugation for 90 minutes at 38,000 r.p.m. (127,000 G). The resulting two pellets were then resuspended one in 3 ml. of distilled water, and one in 3 ml. of Dalton's chrome-osmium fixative; a further short clarifying spin for 15 minutes at 4,000 r.p.m. followed. Once again the supernatants were spun for 90 minutes at 125,000 G and the supernatant fluid was discarded.

The pellets were now carefully rinsed with distilled water and each was finally suspended in 0.5 ml. distilled water. Small drops of each were now placed on electron microscope grids and were dried over calcium chloride in a dessicator. The prepared grids were placed in a shadow casting apparatus and shadowed with gold palladium alloy (60 per cent gold, 40 per cent palladium) at an angle of 15 degrees.

(b) Phosphotungstic acid staining

The purified suspension of measles elementary bodies described above was also stained with phosphotungstic acid by the method of Brenner and Horne (1959) and was sprayed onto electron microscope grids with a "Vaponephrin Nebuliser".

A second approach was to use whole infected cells as the starting material and for this, tissue cultures showing 75 per

cent of the cells involved in syncytial formation were employed. They were detached from the glass either by trypsinisation or by freezing and thawing. The cells were then disintegrated by further freezing and thawing alternately at  $-30^{\circ}\text{C}$  and at  $+37^{\circ}\text{C}$ . After centrifuging for 20 mins. at 5,000 r.p.m., the deposit was suspended in 1 per cent aqueous ammonium acetate. One half of this suspension was mixed with two volumes of 2 per cent phosphotungstic acid adjusted to pH. 7 with  $\text{N}/1$  potassium hydroxide; the other half was emulsified in four volumes of 0.5 per cent phosphotungstic acid in 50 per cent alcohol. The final product was sprayed on to an electron microscope grid as described above.

### Distemper

#### (a) Shadowed preparations

i. The virus was grown in ferret kidney tissue culture, and 80 ml. of the infected tissue culture fluid was purified and preparations made as described before for measles virus.

ii. Virus grown on chorio-allantoic membrane of embryonated hen's egg were also used. Five membranes with large lesions were selected and carefully trimmed close to the lesions. These were then ground in distilled

water, using a Ten-Broeck tissue grinder. The suspension was further disintegrated by freezing and thawing alternately at  $-30^{\circ}\text{C}$  and  $+37^{\circ}\text{C}$ . The suspension was centrifuged for 20 minutes at 5,000 r.p.m. A portion of the supernatant was used for titration and the rest went through the same process of repeated ultra centrifugation; the resultant virus pellet was used for P. T. A. stained and shadowed preparations.

(b) Virus for P. T. A. staining was also obtained from infected ferret spleen by the following method.

The spleen was removed with sterile precautions from a distemper-infected ferret in a moribund state. This spleen was washed, decapsulated, and minced into small fragments, about 1 to 2 m.m. in size. These pieces were repeatedly washed in sterile distilled water until the supernatant was almost colourless. This required about 10 to 12 washings. The sediment from the final washings was ground with four to five volumes of sterile distilled water in a Ten-Broeck grinder. The resultant suspension was clarified by centrifugation for 20 minutes at  $2.4^{\circ}\text{C}$ , in a Spinco Model L. ultracentrifuge, using angle head No. 21 at 6,250 r.p.m. (3,900 G). The middle opalescent portion was carefully pipetted out, further diluted with distilled water. This suspension was further centrifuged using the angle rotor No. 21 at (12,000

r.p.m.). The supernatant was centrifuged once more in a swing-out rotor head No. 39 for 120 minutes at 38,000 r.p.m. (127,000 G). The resultant pellet was resuspended in 6 ml. of distilled water, and centrifuged as before. This pellet from the second centrifugation was used for P. T. A. staining with 0.5% P. T. A. in 50% Ethyl alcohol.

The tissue culture fluids were harvested when maximal cytopathic effects were seen, i. e., 2 to 10 days with amnion cells and 2 to 3 days with HEP-2 cells. At this time most of the affected cells were seen to be detached from the glass surface. The remaining cells were frozen and thawed twice, and pooled with the harvested tissue culture fluid, and centrifuged for 20 minutes at 2000 R.P.M.

Concentration of the virus: 100 ml. of the tissue culture fluid was placed in sterile 'Visking' dialysis tubing and dialyzed against three changes of distilled water at +20°C in 24 to 36 hours. These tubes were then dehydrated, by surrounding them with dry calcium chloride powder, and kept under constant weight at +20°C. Usually, it was necessary to change the powder once in 24 hours. After 24 to 36 hours, the concentrated virus fluid was squeezed in one end of the tubing.



## HAEMAGGLUTINATION

and

## HAEMAGGLUTINATION-INHIBITION TESTS

### Measles antigen

The 'Allan' strain of measles virus was grown in Roux bottles on primary human amnion cell layers and the HEp-2 adapted virus on HEp-2 cells, again in Roux bottles. The tissue culture fluids were harvested when maximal cytopathic effects were seen, i. e., 8 to 10 days with amnion cells and 2 to 3 days with HEp-2 cells. At this time most of the affected cells were seen to be detached from the glass surface. The remaining cells were frozen and thawed twice, and pooled with the harvested tissue culture fluid, and centrifuged for 20 minutes at 2000 R. P. M.

Concentration of the virus: 100 ml. of the tissue culture fluid was placed in sterile 'Visking' dialysis tubing and dialysed against three changes of distilled water at +2°C in 24 to 36 hours. These tubes were then dehydrated, by surrounding them with dry carboxy-methyl cellulose powder, and kept under a constant weight at +2°C. Usually, it was necessary to change the powder once in 24 hours. After 24 to 36 hours, the concentrated syrupy fluid was squeezed to one end of the tubing,

and the fluid drawn into a Pasteur pipette, introduced by puncturing. The inside of the tubing was washed with one or two ml. of sterile distilled water and this was pooled with the original concentrate; thus a final volume of five ml. was obtained from the original 100 ml. of tissue culture fluid. This was preserved frozen at  $-70^{\circ}\text{C}$ .

Rhesus monkey red-blood cells in a modified Alsever's solution were kindly supplied by Dr. Grist of Ruchill Hospital, Glasgow. The cells were washed and suspensions made and used as described by Rosen (1960).

To determine the optimal quantity of red blood cells to be used in the tests the washed packed rhesus cells were diluted in D.G.V. to make concentrations of 2.0, 1.0, 0.75, 0.50, 0.25 per cent. From each concentration 0.4 ml. was allowed to settle in the cups of W.H.O. plastic plates and readings were taken at the end of a half, one, and two hours. A firm and clear-cut button at the end of half and one hour indicated the suitable concentration of cells to be used.

Haemagglutination tests Serial doubling dilutions of the virus were prepared in saline. To each 0.2 ml. of virus dilution an equal volume of 0.5 per cent monkey red blood cells were added, and these plates were incubated at  $+37^{\circ}\text{C}$ ,

room temperature ( $18^{\circ}\text{C}$ ) and at  $+4^{\circ}\text{C}$ . Readings were taken at the end of half and one hour.

A control with cells alone was also put up. The dilution that showed complete haemagglutination was taken as one H.A. unit.

#### HAEMAGGLUTINATION INHIBITION TESTS

Sera were absorbed with kaolin and then with rhesus erythrocytes (Rosen 1960). Serial doubling dilutions from 1 in 2 to 1 in 512 were then prepared with 0.85 per cent NaCl and 0.25 ml. of each dilution was placed in  $2 \times \frac{1}{2}$ " tubes. Measles virus antigen diluted to contain 4 H.A. units in 0.25 ml. was then added to each serum dilution and these virus serum mixtures were mixed and allowed to stand at room temperature for one hour. Volumes of 0.4 ml. of the virus serum mixtures were then transferred to the cups of W.H.O. plates, and to each was added 0.2 ml. of an 0.5 per cent suspension of rhesus monkey red blood cells; readings were taken after one hour at room temperature.

## COMPLEMENT FIXATION TESTS.

In general the method of Bradstreet and Taylor (1962) was used. This technique employs complement preserved by Richardson's method (1941) used at a dilution to give 2 M.H.D. and a suspension of sensitized red cells adjusted to give an accurate 2 per cent reading on an E. E. L. Colorimeter. The optimal concentration of the haemolytic serum and the titre of the complement are determined in a chess-board titration. All the tests are carried out in W. H. O. plastic trays. The diluent used is the Ca - Mg buffered solution of Mayer et al. (1946). Titres are expressed in terms of the reciprocal of initial dilution of the serum used. Sera were inactivated for 30 minutes at 56°C.

### Antigens

Measles. Two antigens were used.

(a) A tissue culture fluid antigen prepared by a modification of the method of Caunt, Rondle and Downie (1961). The Edmonston strain of the Virus was used to inoculate Roux bottles which had been seeded 48 hours previously with HEp 2 cells so that infection occurred just prior to the formation of a complete monolayer. A dose of 5 ml. of virus containing  $10^4$  T.C.I.D./50, per 0.1 ml. was used for each bottle. The maintenance medium used was 30 per cent bovine amniotic



fluid in Hanks' solution. When a marked cytopathic effect was seen in all the bottles, usually 4 - 5 days later, the tissue culture fluids (100 ml. from each bottle) was harvested. The cells remaining adherent to the glass were removed by freezing and thawing and were added to the bulked tissue culture fluids and the whole was clarified in the centrifuge for 10 minutes at 2,000 r.p.m. The supernatant fluid was dialysed for 36 hours against three changes of distilled water at 2°C. Next the fluid was concentrated by surrounding the dialysis tubing with carboxy-methyl cellulose powder as described above in the method of antigen preparation for the haemagglutination inhibition test. The final product, usually about 5 ml. of a syrupy fluid constituted the antigen used in the tests. The optimal dilution of the antigen for use in the tests was estimated in a chessboard titration against a human convalescent serum ; in practice this dilution was usually 1 in 8 or 1 in 16.

(b) A mouse brain antigen was prepared by the sucrose-acetone extraction method employed by Clarke and Casals (1958) in the serological studies of Arboviruses.

Some 25 suckling mice, 48 hours old, were inoculated intra-cerebrally with 0.01 ml. of the Edmonston strain of the virus which had been adapted to the mouse (see p.185. ).

Animals developing spastic paralysis or found moribund (usually 5 - 7 days after inoculation) were killed and the brain tissue was collected by aspiration using a method described elsewhere. The pooled brain tissue was homogenised with 4 volumes of chilled 8.5 per cent sucrose and was then centrifuged for 5 minutes at 1,800 r.p.m. Both the deposit and the supernatant fluid were separately treated by adding acetone to the original volume and were placed for one hour in an ice bath. Both were centrifuged again as before and after removal of the supernatant, the sediment was dried in vacuo in a dessicator over calcium chloride. The dried powder was reconstituted before use by adding a volume of saline equal to 0.4 of the total volume of the homogenate used; it was held overnight at 2°C and centrifuged for 30 minutes in the cold at 10,000 r.p.m. The supernatant constituted the antigen used in the tests and was titrated in the same manner as the tissue culture antigen. It was possible to store the reconstituted antigen at -70°C for two months without loss of titre.

#### Distemper

An attempt was made to prepare a complement fixing antigen using allantoic membranes infected with the Epivax strain of the virus. The sucrose-acetone extraction method

was employed but the final product proved to be highly anti-complementary. Further attempts to overcome this difficulty were unsuccessful and the method was discarded. A second attempt to prepare an antigen from the infected allantoic membranes in which the virus was concentrated and purified by cycles of differential centrifugation was equally unsuccessful and the product was again highly anti-complementary.

However, it was found possible to use a suspension of splenic tissue taken from infected ferrets and antigens of this type were employed successfully. The spleen was taken from a moribund ferret 10 - 14 days after subcutaneous inoculation with the "wild 2" strain of the distemper virus. After weighing the tissue was homogenised to make a 10 per cent W/V suspension in saline. The optimal dilution for use in complement fixation tests was determined in a chess board titration against a convalescent canine serum.

## NEUTRALIZATION TESTS

### Measles

The virus was grown in HEP-2 cells and harvested cells, together with the tissue culture fluid, were frozen and thawed and clarified by spinning. The supernatant formed the stock virus used in the tests. This was titrated by diluting in serial ten-fold steps in Medium 199. 0.5 ml. per tube was inoculated, and three tubes for each dilution were used. The tubes were observed for 7 to 10 days. Maintenance medium was changed twice weekly.

The virus stock was diluted to contain 200 TCID<sub>50</sub> in 0.5 ml. Serum was inactivated for 20 minutes at 60°C and doubling dilutions were made. 0.5 ml. of stock virus (200 TCID<sub>50</sub>) and 0.5 ml. of diluted serum were incubated at room temperature for 1 hour; and 0.5 ml. of this mixture was inoculated to 4 tubes for each dilution.

The tubes were examined 24 hours later for any contamination or serum toxicity, and were watched daily for 10 days. Maintenance medium was changed every third or fourth day. The serum neutralisation titre was the highest dilution of the serum which protected 50 per cent of the cultures.



### Distemper

The avianised strain (EpiVax) of distemper virus, was inoculated on to the chorio-allantoic membrane of 6-day old embryonated eggs and incubated at 37° for a further 7 days; when opened, typical lesions were seen. The membranes were trimmed as close to the lesions as possible, washed in sterile P. B. S. with antibiotics (100 units of Penicillin and 100 mg. Streptomycin per ml.), and a fine suspension prepared with a Ten-Broek tissue grinder. An equal volume of medium 199 was added. This suspension was clarified by spinning for 10 minutes at 4,000 R. P. M. The supernatant was titrated and the infectivity titre ( $EID_{50}$ ) was calculated.

### Serum

Two human convalescent sera, and two canine distemper sera, as well as the type antisera of rabbit for measles and horse for distemper were also used.

The virus was diluted to contain 500  $EID_{50}$  per 0.5 ml. of the inoculum and doubling dilutions of sera were made in 199. 0.2 ml. of the virus mixture and 0.2 ml. of the diluted sera were well mixed, incubated for 1 hour at room temperature (22°C). 0.1 ml. of this mixture was inoculated into each egg on the chorio-allantoic membrane, using 3 eggs for each dilution. The embryos used had been incubated at

IMMUNE ELECTROPHORESIS

39°C for 6 to 7 days, prior to inoculation. The inoculum was tested for bacterial sterility as well.

Kohn) Mark II was used.

Technique was also evolved in which precipitation was studied by immune electrophoresis. It is described together with results on page

PRECIPITATION REACTIONS

Precipitation reactions in agar-gel was studied with measles virus and distemper virus with the appropriate antisera. Both macro and micro methods were used. The tests were carried out in tubes as well. These methods, their modification and the results obtained are reported on page 102.

IMMUNE-ELECTROPHORESIS

SHANDON - Universal Electrophoresis apparatus (after Kohn) Mark II was used.

Technique was also evolved in which precipitation was studied by immune electrophoresis. It is described together with results on page

PRECIPITATION IN AGAR-GEL

Precipitation reaction in agar-gel was studied with measles virus and distemper virus with the appropriate antisera. Both macro and micro methods were tried. The tests were carried out in tubes as well. These methods, their modification and the results obtained are described on page 198

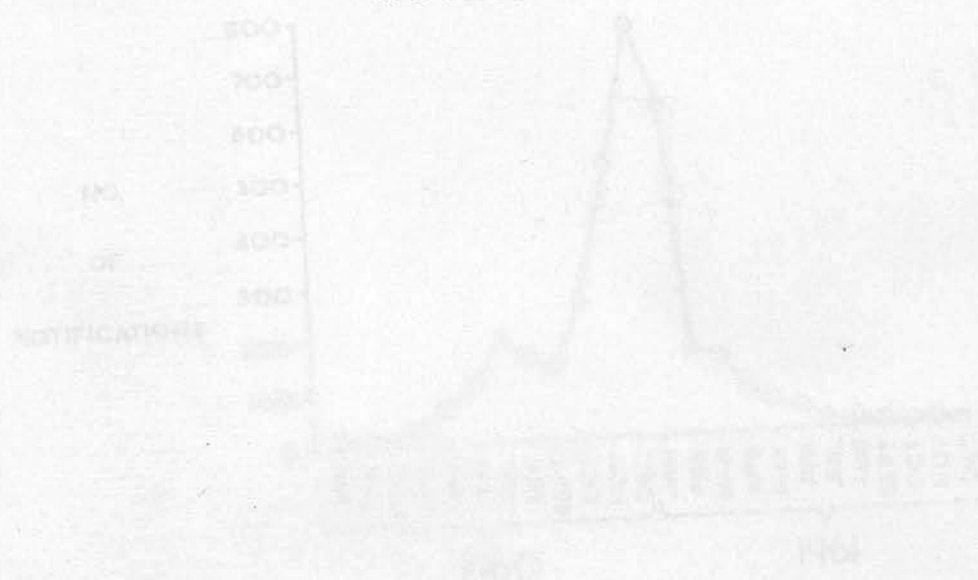
## SECTION I

### Isolation of the virus and serological studies in clinical cases.

The biennial epidemicity of measles in the City of Birmingham is reflected in the figures for the total notification each year. These notifications are made only for the first case occurring in each household and this cannot give any precise data on the incidence of the disease. Nevertheless the trend is seen in the following table:

1956	2.531	notificazioni
1957	1.252	"
1958	1.733	"
1959	1.257	"
1960	2.282	"
1961	1.202	"

## RESULTS



These figures show the epidemicity of measles in the City of Birmingham. Figures for the years 1956-1961 are taken from the Birmingham Health Department. Only the first case in each household is notified.

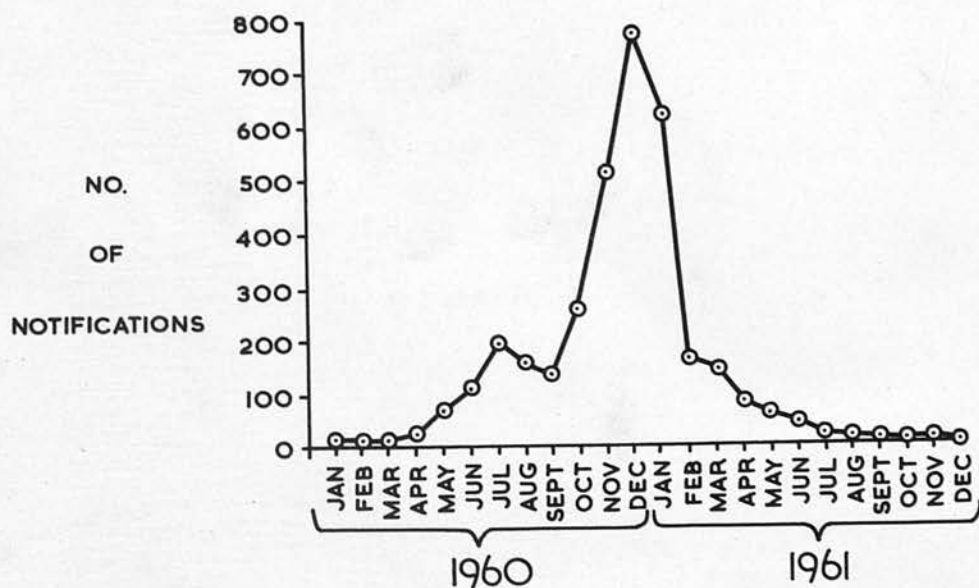


## SECTION I

### Measles     Isolation of the virus and serological studies in clinical cases.

The biennial epidemicity of measles in the city of Edinburgh is reflected in the figures for the total notification each year. These notifications are made only for the first case occurring in each household and this cannot give any precise data on the incidence of the disease. Nevertheless the trend is seen in the following series:

1956	2,631	notifications
1957	1,284	"
1958	1,753	"
1959	1,257	"
1960	2,288	"
1961	1,202	"



Graph showing the monthly notifications of measles 1960-61 in the City of Edinburgh - Figures from Annual Reports of Public Health Department. Only the first case in each household was notified.

### ISOLATION OF VIRUS STRAINS

Cases of measles, both with fully developed rashes, and a few suspected contacts in prodromal stages were admitted to the Edinburgh City Fever Hospital. From some of these cases, the following clinical material was collected within an hour or two after admission.

- i. Throat. Pharyngeal and nasal swabs
- ii. Blood. a. Venous blood  
b. Capillary blood
- iii. Urine. From one case. The first morning specimens were collected in sterile containers which contained 2 to 3 ml. of sterile horse sera (inactivated) and antibiotic mixture. This was kept stored in the refrigerator immediately after voiding, until it was collected by the laboratory staff.
- iv. Pieces of lung and brain - removed at post-mortem examination of case that had been diagnosed clinically as a case of measles encephalitis.

#### Isolation of strains:-

Throat swabs Dry sterile throat swabs, were

rubbed on the pharyngeal and tonsillar areas of the throat. The swabs were then broken off into Bijou bottles containing 3 ml. of the following mixture:-

- (a) Parker's "199".
- (b) Sterile inactivated calf serum to give a final concentration of 5 per cent.
- (c) Antibiotics in the following proportions:-

Sodium penicillin.	1,000 units per ml.
Streptomycin hydrochloride.	500 mg. per ml.
Mycostatin.	100 mg. per ml.

These Bijou bottles were kept stored in the refrigerator (+4°C) for about 4 hours.

The swabs were then squeezed out and the fluid clarified by centrifuging for 10 minutes at 1000 R. P. M. in an M. S. E. centrifuge. Half to one ml. of this clarified supernatant was then inoculated onto young grown out monkey kidney, human amnion cell, and Hela cell monolayer and, in one case, to human embryonic kidney cell monolayer. These tubes for inoculation were prepared as follows:-

The maintenance medium was poured out and the cell layer washed once with sterile P. B. S. The inoculum was allowed to remain in the tube for two

to three hours at room temperature (18 to 22°C), after which it was poured off. The cells were washed once with maintenance medium which was then replaced by 1 to 1.5 ml. of fresh maintenance medium. The inoculated and the control tubes were then rolled, and examined every day for three weeks. The maintenance medium was changed every third or fourth day in all except the amnion cells, for which it was done once a week.

Blood Both venous and capillary blood was used to inoculate the tissue culture tubes.

(a) Venous blood From previous experience of isolating Rickettsiae (Kalra and Rao, 1950) and, on the assumption that the leucocytes carried the virus (Papp 1956), attempts were made to inoculate only the leucocytes, discarding as much as possible of the serum and the red blood cells.

5 to 10 ml. of venous blood from cubital veins were drawn in pre-cooled syringes that had been rinsed in heparin solution (20 mg/per cent) and immediately transferred to pre-cooled centrifuge tubes. These were centrifuged at +4°C for 10 to 12 minutes at 2,000 R. P. M. The buffy layer with



the minimum amount of red blood cells and plasma, was sucked into a capillary pipette and used as the inoculum. 0.25 ml. to 0.5 ml. were inoculated into each tube.

In three cases, whole blood, without heparin, was directly inoculated into tissue culture tubes at the bedside, the inoculum per tube being half to one ml. of the blood.

(b) Capillary blood In another two cases of small children, less than a year old, it was found difficult to draw sufficient blood by venipuncture. Hence, the following method was used: the heels were well cleaned with alcohol and ether, which was allowed to evaporate, and were then washed with sterile saline twice. The heel was then covered with a piece of sterile gauze leaving a small portion bare. A deep puncture was made in this exposed part with a lancet and 4 to 5 drops of blood were collected directly into the prepared tissue-culture tubes, using sterile precautions. However, in both cases no strains were isolated.

Urine The urine was neutralized by the addition of 4.4 per

cent of sod. bicarb. so that the pH was brought up to 7 to 7.4. It was then centrifuged for 10 mins. at 2,000 R. P. M., and the deposit was used as the inoculum. After two hours, the inoculum was washed off and fresh maintenance medium added.

#### A fatal case of measles encephalitis

The case history, post mortem findings and the histo-pathology of the case is given below.

D-. G-. Age  $7\frac{1}{2}$  years: On 9th January, 1961, the child became feverishly ill, had frequent watery stools and vomited. A rash appeared during the day. On 12th January, she was seen by her doctor who diagnosed measles. When the doctor made his return visit the following day, the child was semi-comatose and she was transferred to the City Hospital.

On admission (4 days after the appearance of the rash): She had a temperature of  $102^{\circ}\text{F}$ , a pulse of 116, and her respirations were 32 per minute. The skin was covered almost completely with a purpuric morbilliform rash and the pharynx was full of viscid muco-pus. There was slight neck rigidity but Kernig's sign was negative. She had fluctuating

periods of consciousness. The cerebrospinal fluid contained 106 cells per cu. mm., 90 per cent of which were lymphocytes, but no other abnormality. A radiograph of the chest showed early bronchopneumonic change on the right side.

The general condition rapidly degenerated, the pulse rate rose to 208 per min, the temperature to 105°F, and Kernig's sign became positive with the limbs becoming markedly spastic.

The patient died three days after admission and 7 days after the onset of the illness.

An autopsy was carried out on the day after death and the following abnormalities were described by the pathologist.

The entire body was covered with an erythematous rash. Both lungs were oedematous and completely consolidated. Widespread bronchopneumonia was found in all the lobes of the left lung while the right lung was congested with the lower lobe collapsed. The trachea and bronchi were acutely congested and contained much muco-pus.

The meninges were markedly congested but there was no evidence of meningeal adhesions. After fixation, the brain showed some swelling of the hemispheres and congestion of the cortical vessels, especially those in the

white matter of the centrum ovale on each side.

Histological sections from all areas showed a perivascular inflammatory reaction composed of neutrophil granulocytes, and mononuclear cells, probably plasma cells and proliferative histiocytes. There was evidence of demyelination in the areas of inflammation. The appearances were those of acute disseminated encephalomyelitis (post-infectious encephalopathy).

Histological examination of the heart, kidney, intestinal tract, thyroid, pituitary, adrenal and parathyroid glands showed no abnormality. The lung was the site of bronchopneumonic changes only. Despite a prolonged search no mulberry giant cells (Finkeldey and Warthin cells) which are diagnostic of measles could be found in any of the organs.

Portions of lung and of the frontal lobe of the brain were taken during the post mortem and were brought to the virology laboratory where they were placed in medium 199 with added antibiotics and stored at  $-32^{\circ}\text{C}$ .



TABLE TO SHOW THE ISOLATIONS OF MEASLES  
VIRUS FROM HUMAN CLINICAL MATERIAL

<u>Patient's Initials</u>	<u>Age in Years</u>	<u>Time of collection in relation to rash. In hours</u>	<u>Material collected</u>	<u>Cell layer and passage history.</u>	
C. S.	6	+ 36	T. S. & V. B.	Mk. 0	Am. 0 (whole blood)
H. D.	1½	+ 24	T. S.	0	0
P. O. B.	10/12	+ 48	T. S. & H. P. B.	0	0 (Heel prick blood)
A. B.	2½	- 24	T. S. V. B.	P <sub>1</sub> P <sub>1</sub>	0 0 (whole blood)
P. M.	1	+ 48	T. S. + H. P. B.	0	0
M. T.	2½	0 +	T. S. V. B.	P <sub>2</sub> P <sub>1</sub>	0 0 (whole blood)
P. O.	3	+ 24	T. S. + V. B.	0	0
J. A.	4	+ 6	T. S. V. B.	P <sub>1</sub> P <sub>1</sub>	A <sub>1</sub> (Leuco- cytes).
J. H.	6	+ 48	T. S. + V. B.	0	0
L. H.	9	+ 6	T. S. V. B.	P <sub>2</sub> P <sub>1</sub>	0 A <sub>1</sub> (Leuco- cytes)
M. G.	12	+ 24	T. S. V. B. Urine Dys. 1, 2, 3, 4	0 P <sub>2</sub> P <sub>1</sub>	0 0 (Leuco- cytes) 0 (3rd & 4th day. Urine only)
D. S.	7	+ 5 days	Brain and Lung	0 P <sub>1</sub> *	0 0

TABLE TO SHOW THE ISOLATIONS OF MEASLES

VIRUS FROM HUMAN CLINICAL MATERIAL (CONT)

<u>Patient's</u> <u>Initials</u>	<u>Age</u> <u>in</u> <u>Years</u>	<u>Time of</u> <u>collection</u> <u>in relation</u> <u>to rash</u> <u>In hours</u>	<u>Material</u> <u>collected</u>	<u>Cell layer and</u> <u>passage history</u>	
L. J.	12	+24	T. S. & V. B.	Mk. 0	Am. 0
M. S.	13	+24	T. S. & V. B.	0	0
L. S.	8	+10	T. S. & V. B.	0	0

T. S. = Throat swab.

0 = No isolation. 3 passages made.

V. S. = Venous blood

$P_1$  &  $P_2$  = isolation in  
passage 1 or  
passage 2.

H. P. B. = Heel prick blood.

$A_1$  &  $A_2$  = do. do. in  
amnion cells.

All CPE seen 5 to 13 days after inoculation.

\* Isolation in human embryonic kidney monolayer.

Neutralization test carried out in Hep-2 cell monolayer

Virus: 0.5 ml. to contain 200 TCID<sub>50</sub>

Serum: Doubling dilutions in 199.

Period of incubation - 1 hour at room temperature 18°C

<u>Sera</u>	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1:128	1 : 256 (semi dil)
<u>Human Measles</u>								
M.G. (ac).	4/4	4/4	4/4	4/4				
(con).	0/4	0/4	0/4	0/4	0/4	0/4	0/4+	(2/4)
J.A. (ac).	4/4	4/4						
(con).							0/4	(3/4) to go higher.
M.D. (con).					0/4	0/4	0/4	(0/4) to go higher.
M.R. (con).			0/4	0/4	2/4	4/4		
<u>Dog.</u>								
<u>Convalescent</u>								
1	0/4	3/4	2/4	4/4				
2	4/4	4/4	4/4	4/4				
3	(0/4)	4/4	4/4	4/4				
4	0/4	(2/4)	2/4					
<u>Ferret (Measles)</u>								
1	0/4	0/4	3/4	4/4				
2	0/4	4/4	4/4					
<u>Distemper</u>								
1	4/4	4/4	4/4					
2	4/4	4/4	4/4					

Numerator = Number of tubes showing CPE.

Denominator = Number of tubes inoculated.

# RESULT OF COMPLEMENT FIXATION TESTS

with acute and convalescent human sera.

Name of Pt.	Time of collection in relation to rash - in days	Reciprocal of comple- ment fixing titres with antigens of	
-------------	--------------------------------------------------------	-----------------------------------------------------------------	--

		Measles	Distemper
--	--	---------	-----------

AB *	-1	neg.	neg.
	10	64	neg.
LH *	1	neg.	neg.
	9	8	neg.
MS *	1	neg.	neg.
	11	8	neg.
JH *	2	neg.	neg.
	8	8	neg.
MG *	-1	neg.	neg.
	12	64	neg.
	21	512	8
JA	1	neg.	16
	10	16	16
	24	64	32
TG	10	8	neg.
N O'M	10	4	neg.
RP	8	8	neg.
MR	11	32	neg.
Human Gamma ) globulin fraction II )	-	256	8

\*Strains  
isolated



Eight batches of gamma globulin, prepared from the blood of adult blood donors, at approximately monthly intervals, during the year 1962, in the "Blood Transfusion Service" at the Royal Infirmary, Edinburgh were tested for their activity against measles virus.

As all the eight, proved highly anticomplementary, no complement-fixation tests could be carried out. However, a neutralization test was carried out against TCID 200/0.5ml. of measles virus grown in Hep-2 cells.

The titre of neutralizing antibody in seven of the eight batches, was in the range of 256 to 512. One batch gave a lower figure of 1:128. It should be added that 1962, the year during which the blood used was collected, was not a "measles" year.

## SECTION II

### Studies of the effects produced by the measles virus on cells growing in tissue culture

In the following experiments the effects of the measles virus on several host cell systems was observed under varying cultural conditions. The objective was to find a practical method in which a host cell system could be obtained in which to compare the growth of both the measles and distemper viruses. Success was obtained with dog kidney cells and with ferret kidney cells but kidneys from these two species were never available on a scale sufficient to provide enough cultures for comparative growth studies. It was found that the kidney cells from adult and elderly dogs sent for destruction to the small animal clinic at the Royal Dick Veterinary College were unsatisfactory in two respects; they often failed to grow out into satisfactory monolayers despite the use of a wide variety of culture media; and also, even when good monolayers were obtained, the cytopathic effect of the measles virus on them was uncertain and irregular in its appearance. Furthermore the presence of indigenous canine viruses (canine distemper and canine hepatitis) could seldom be excluded. On infrequent occasions

litters of young healthy puppies were brought to the clinic for euthanasia and the kidneys of these animals were extremely satisfactory but the supply of them was so irregular that it was impossible to rely on them.

The colony of ferrets established in the departmental animal house was too small to be able to sacrifice animals on any but infrequent occasions.

Other host cell systems had therefore to be sought and in carrying out this work, the opportunity was taken to observe by the methods of light microscopy the chronological sequence of events that occurred after infection. Unstained and unfixed preparations were examined by low power microscopy and by phase contrast microscopy; conventional staining methods and staining with fluorescent acridine orange using cover-slip cultures after fixation were also employed.

From the foregoing experiments it can be seen that the human amnion cells and the HEP 2 line could be relied on for regular supply and that these systems provided satisfactory virus yields. Human embryos could only be obtained on very rare occasions at approximately 6-month intervals and the line of lung cells came into my hands very late in the course of this work.

Although the measles virus was successfully adapted

to both calf kidney cells and the HeLa cells it transpired later that the distemper virus did not multiply in these host cells.

While monkey kidney tissue cultures were satisfactory in many respects the frequency with which the supply was found to carry simian foamy agents was so great (often three out of four incoming supplies were affected) that their use was precluded. Furthermore the possibility that the cells also carried a monkey measles virus (e.g. M.I.N.I.A. of Ruckle) had also to be borne in mind.

#### Human amnion cells, monolayer

A total of 57 amniotic membranes were processed. At first in the placenta obtained from normal deliveries, the amnion cells showed a non-specific degeneration (toxic) even before the formation of the monolayer. This was attributed to the disinfectants used coming into contact with the placenta. Later, as far as possible only the placenta from Caesarian sections were used.

By using a trypsin-pancreatin mixture (Faul 1963) in calcium magnesium free R. D. S. instead of the trypsin only, much higher cell yields were obtained.

The amniotic membrane from a placenta was stripped



### Monkey kidney cell monolayer

Primary monolayers from Glaxo laboratories and from M. R. C. were used. They were versenised, and distributed into tubes and bottles, as required. The cells received from Glaxo Laboratories were almost invariably contaminated with the foamy agent, and therefore had to be stopped. Those received from M. R. C. were better but even they had to be carefully watched. However, the monkey kidney cells were used only for primary isolation of measles virus and for maintenance of one strain for 8 passages.

### Human amnion cells, monolayer

A total of 67 amniotic membranes were processed. At first in the placentae obtained from normal deliveries, the amnion cells showed a non-specific degeneration (toxic) even before the formation of the monolayer. This was attributed to the disinfectants used coming into contact with the placenta. Later, as far as possible only the placentae from Caesarian sections were used.

By using a trypsin-pancreatin mixture (Paul 1960) in calcium magnesium free P. B. S. instead of the trypsin only, much higher cell yields were obtained.

The amniotic membrane from a placenta was stripped,

washed and divided into two approximately equal portions. One portion was processed with the trypsin-pancreatin mixture, and the other in Trypsin alone, as described under methods (page ) and under identical conditions.

Table showing the cell yield per Amnion processed by the two methods described

(Figure expressed in millions)

Cells per amnion in millions		
	Trypsin + Pancreatin Mixture	Trypsin alone
1	150	122
2	185	126
3	124	80
4	180	105
5	210	116
6	186	126
Average yield	155.833	112.5
1	243	153
2	266	175
3	162	122
4	182	116
Average yield	198.25	141.5

Same day processing

Overnight processing

## CYTOPATHIC CHANGES OBSERVED

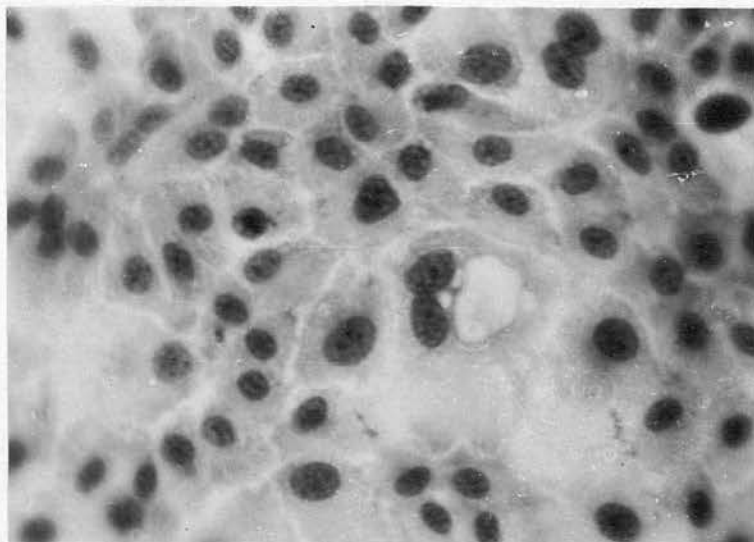
### Measles virus in human amniotic cells.

Two types of cytopathic effects were seen. The first could be described as the typical giant cell formation, first described by Enders and Peebles (1954) and later by several other workers (Ruckle 1957).<sup>a</sup> These giant cells were observed as early as the 6th day in the later passages, and by the 12th to 15th day, large syncytia, involving 80 per cent or more of the cell surface could be seen. The affected part of the cell-layer would ultimately fall off from the glass surface. This effect was seen when the virus was continuously passaged or when very fresh virus stored only for short periods was used as the inoculum. (Phase contrast photograph 123-5).

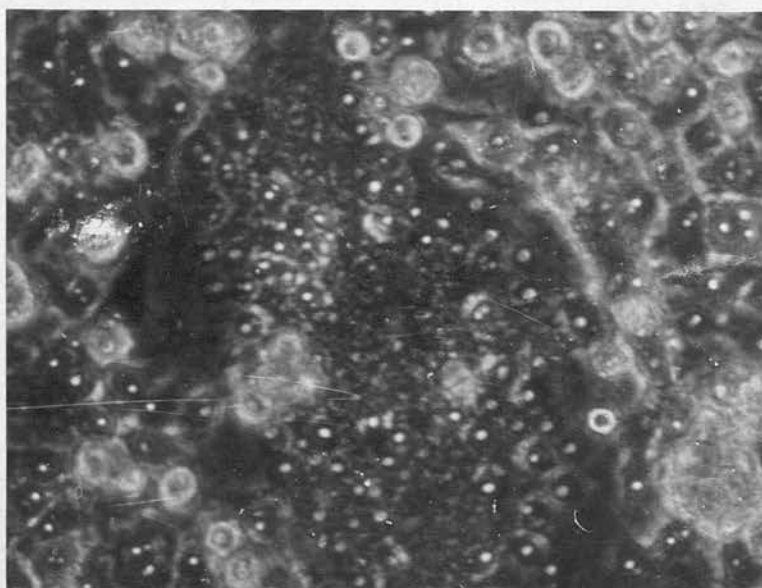
The second type of cytopathic effect was seen when the tissue culture fluid and the cells used as the inoculum had been stored for over six weeks. Eleven days after inoculation a cytopathic effect, completely different from the above, was seen. The individual cells appeared enlarged and contained sometimes 2 to 5 nuclei. The corners of the cells were drawn out into long strands consisting of cytoplasm, giving a star-like appearance. The different cells were joined to each other by fine strands. Sometimes an intra-cytoplasmic

inclusion body (acidophilic) could be seen in the base of these strands, in stained preparations. In general, this gave an appearance of a fine net-work. This type of effect has also been described in several other measles in infected cell systems. (Reissing, Black & Melnick, 1956, Milanovic, Enders and Mitus (1957), Frankel and West, 1958; Seligman and Rapp, 1959., <sup>Odde</sup> Flaccomio and Sinatra, 1961).

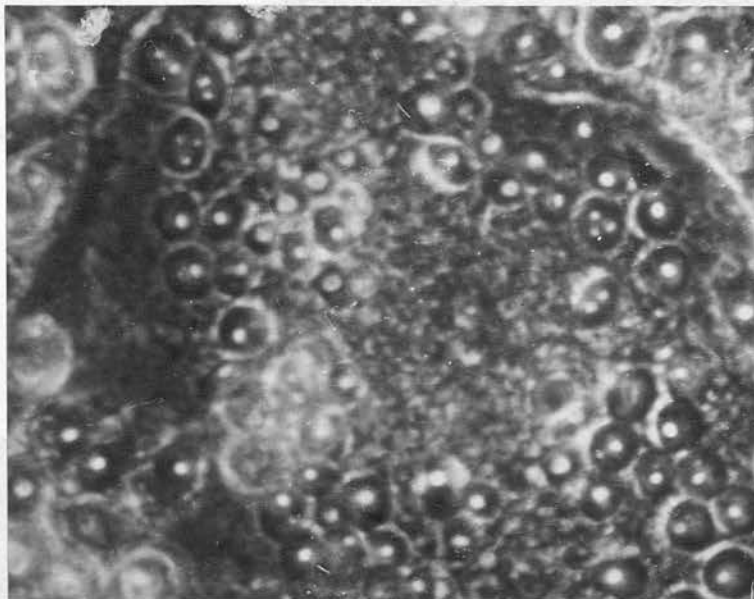




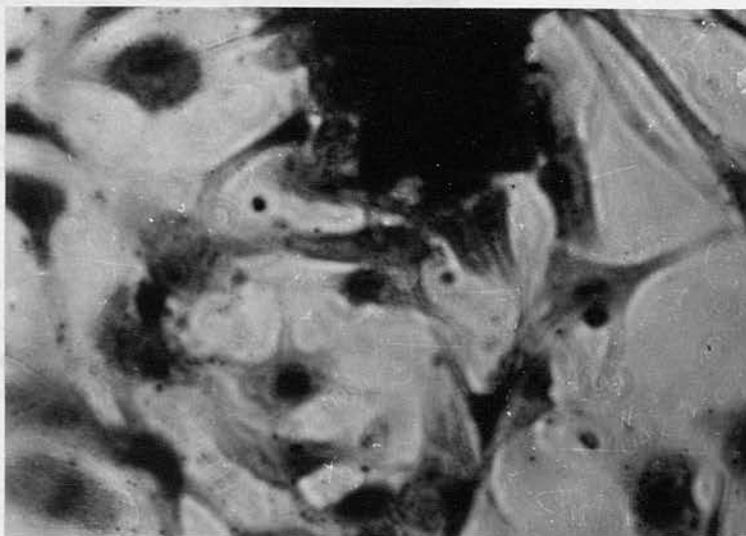
An uninoculated human amnion cell monolayer -  
12 days growth. x40.



Human amnion cell monolayer. Inoculated with  
fresh measles Virus (Allan Strain), showing  
syncytial formation. 9 days after inoculation.  
Note the uniform distribution of Nucleii. Test  
tube preparation. Phase contrast (darkfield) x40.



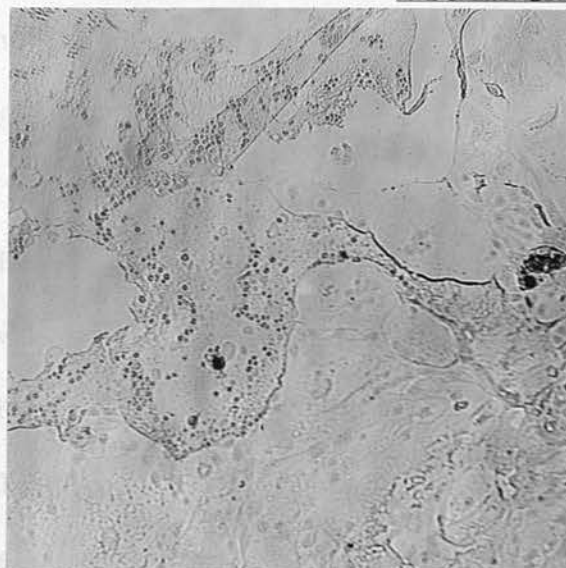
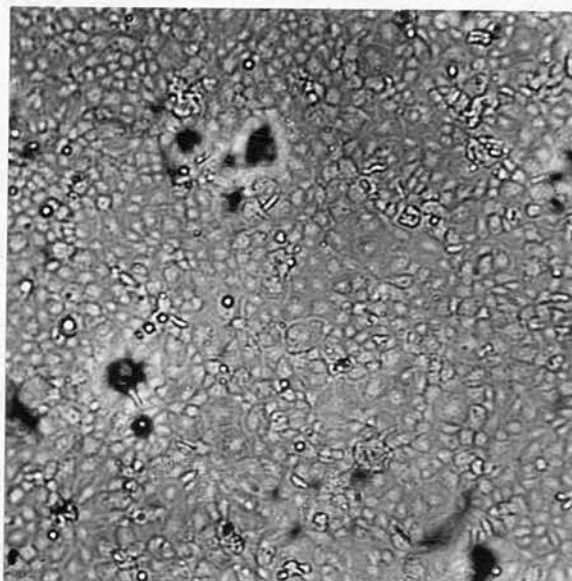
Human Amnion cell monolayer. Same as in the previous photograph but at a higher magnification. (Phase contrast - darkfield) x80.



Human Amnion cell monolayer inoculated with the Virus stored at  $-30^{\circ}\text{C}$ . for 6 weeks. Note the "stranding" of cells. Test tube preparation Negative phase contrast x80.

Human amnion cells  
x70

Normal control



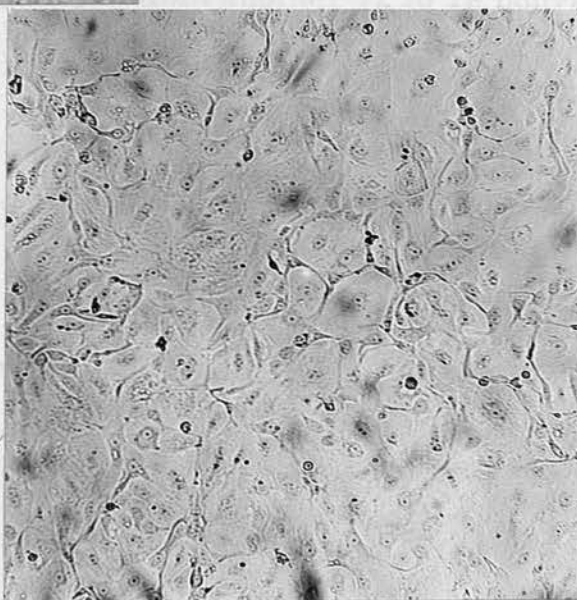
Human amnion cells  
x85

Syncytial effect produced  
by Allan strain of  
measles virus after  
day.

Virus was used fresh  
(unstored)

Human amnion cells  
x70

12 days after inoculation  
with Allan strain of  
measles virus which had  
been stored at  $-20^{\circ}\text{C}$ .



Cytopathic changes seen in HEP-2 cells

Toolan in 1954 established the line of HEP-2 cells, and Black (1956) adapted the Edmonston strain of measles virus to grow in them. The HEP-2 cells were grown in Eagles' basic medium made in Hanks' BSS with 10 per cent calf serum and maintained with Eagles' basic medium made in Hanks' with 2 per cent calf serum. The maintenance medium was changed every third day.

The Edmonston strain of HEP-2 adapted virus was inoculated into the grown out monolayer. The first C. P. E. was seen in unstained tubes was after 48 hours, when the inoculum was quite high (0.5 ml. of T. C. F. titre  $10^{5.2}$ /ml). The C. P. E. seen was that of cells in located areas appearing swollen, enlarged and granular; within another 12 to 18 hours, small giant cells with 5 to 12 nucleii were seen. These rapidly increased in size to form large syncytial masses. The nucleii were closely packed with very little cytoplasm between them. Within 7 to 10 days the entire cell sheet became involved and the affected cells were detached from the glass.



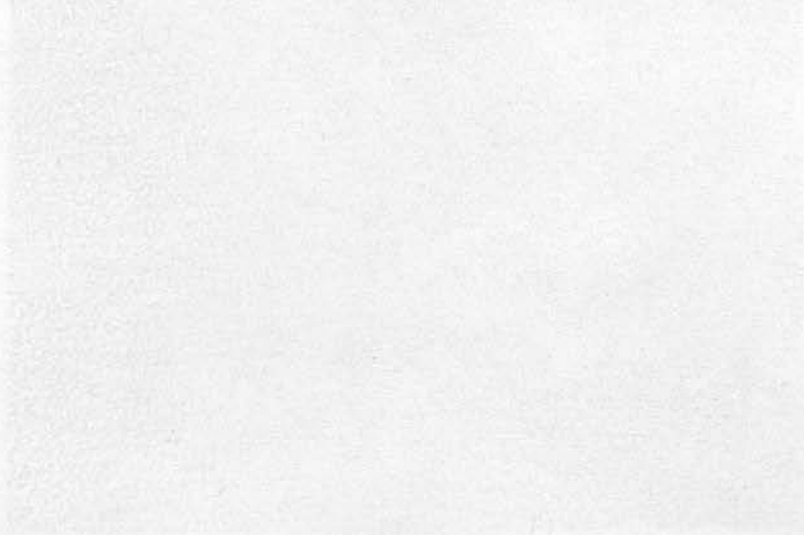
### Growth of measles virus in HeLa cells

A strain of measles virus grown in human amniotic membrane <sup>was</sup> ~~were~~ adapted to grow in HeLa cells.

The Allan strain of freshly isolated measles virus was grown in human amniotic cells till the maximal cytopathic effect was seen. The maintenance fluid was then harvested and the tubes drained. HeLa cells in a concentration of 100,000 /ml. in the growth medium were introduced and the tubes were kept stationary for 48 hours, when a monolayer of HeLa cells formed over the existing amnion cells completely covering them. After 72 hours the growth medium was changed to maintenance medium (Eagles made with Earle's saline and 3 per cent calf serum). In about 5 days cytopathic effects were seen in the HeLa cells and by the 10th day, it appeared that most of the cell sheet had been involved.

The tissue culture fluid and all the cells at this stage were harvested, clarified by spinning for 5 minutes at 4,000 r.p.m. and the supernatant then inoculated fresh grown out monolayers of HeLa cells. The cytopathic effects in these sets of tubes were first seen in about 5 days' time as rounded granular cells which, when situated in the centre of monolayer, coalesced to form small giant cells, but if at

the margin had irregular drawn out processes resembling the "strand forming" cells of Seligman and Rapp (1959). Further passages resulted in true syncytial formation; an effect which was neutralised by known anti-measles serum.

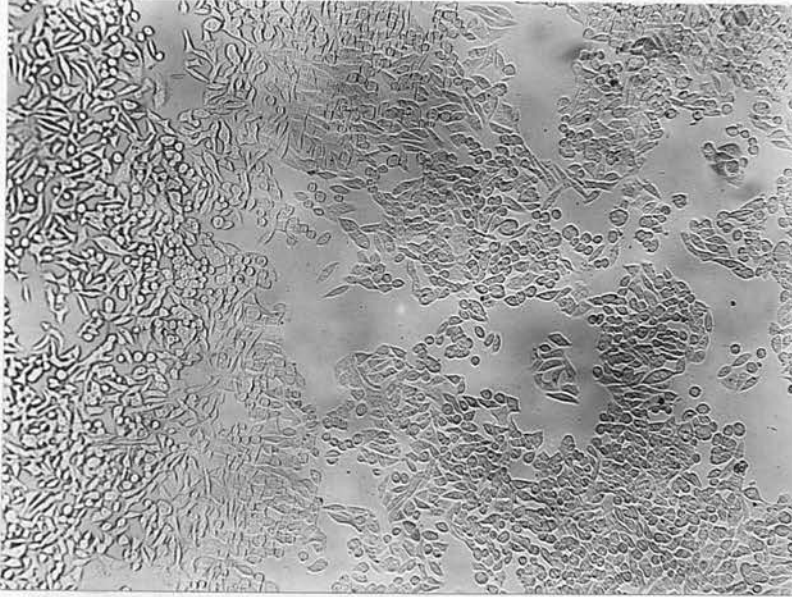


Normal Hela Cells (70)

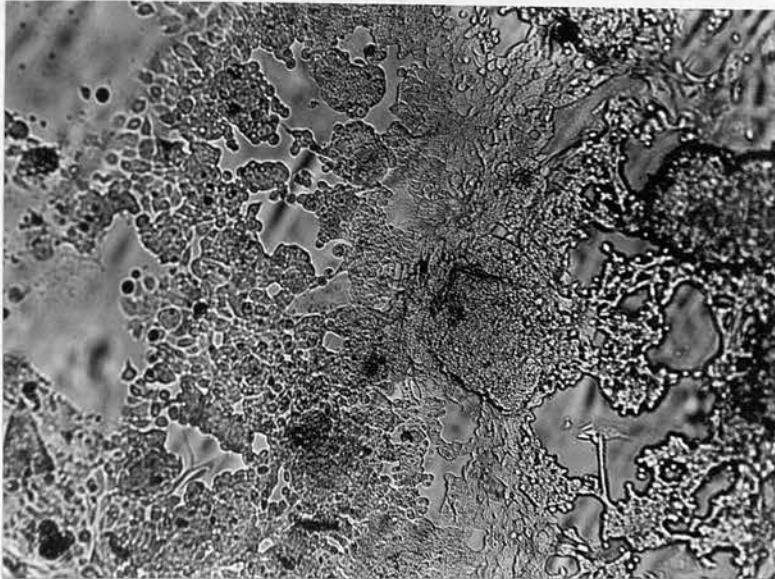


A micrograph of measles virus (70) showing extensive syncytium formation due to infection with the measles virus. Normal Hela cells (uninfected above) were removed from the glass by trypsinization and were seeded onto the surface of the infected cells. Photograph taken 5 days after seeding.

The following photographs illustrate the steps used for the rapid adaptation of the wild 'Allan' strain of the measles virus to growth in HeLa cells.



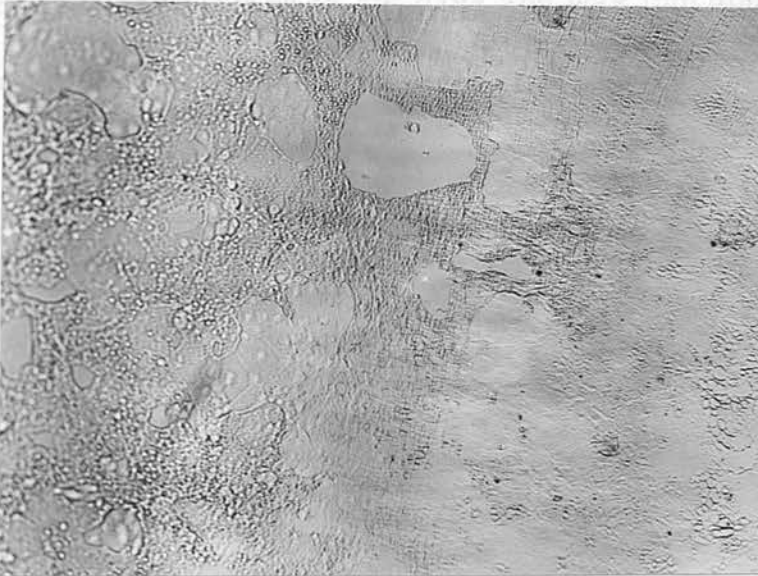
Normal HeLa Cells x70



A monolayer of monkey kidney cells (x70) showing extensive syncytium formation due to infection with the measles virus. Normal HeLa cells (illustrated above) were removed from the glass by trypsinisation and were seeded onto the surface of the infected cells. Photograph taken 5 days after seeding.

### Human Kidney monolayer

Mixed human embryonic kidney tissue was received from Professor A. MacDonald of Aberdeen. This was trypsinized and dispensed into tubes (250,000 cells/ml.)



HeLa cells (x85) showing syncytial formation four days after inoculation with the measles virus. The infecting virus was obtained directly from the mixed cell culture seen in the last photograph and comprised cell free tissue culture fluid prepared by freezing and thawing.

The cells were then washed with 199, so that all traces of the inoculum (especially brain material) was removed. It was replaced by 2 ml. of medium 199 containing 2 per cent horse serum. The tubes were observed every day and the medium was changed twice a week.



### Human kidney monolayer

Minced human embryonic kidney tissue was received from Professor A. MacDonald of Aberdeen. This was trypsinised and dispensed into tubes (250,000 cells/ml.) A monolayer was formed in six days and was inoculated with suspensions of human tissue prepared as follows:

Pieces of brain and lungs recovered at P.M. from the case of measles encephalitis were weighed and a 20 per cent weight/volume suspension prepared using a Ten-Broeck grinder. This suspension was further clarified by spinning at +4°C for 5 minutes at 1500 R.P.M.

Tubes with good monolayers were selected and six tubes for each inoculum were used. The tubes were first washed with medium 199, and then inoculated with 0.5 ml. of the tissue suspensions. Cells and inoculum were allowed to remain in contact for two hours at room temperature.

The cells were then washed with 199, so that all traces of the inoculum (especially brain material) was removed. It was replaced by 2 ml. of medium 199 containing 2 per cent horse serum. The tubes were observed every day and the medium was changed twice a week.



Table showing time of appearance of the measles C.P.E. in  
Fetal lung cell human embryonic kidney cells

<u>Day of observation</u>	<u>Lung emulsion</u>		<u>Brain emul-</u> <u>sion</u>
	C.P.E.	No. of tubes showing CPE	CPE.
1	0		0
2	0		0
3	0		0
4	0		0
5	0		0
6	+	1/6	0
7	+	3/6	0
8	+	3/6	0
9	++	4/6	0

Tubes were observed for 3 weeks

The cytopathic effect was first seen at the margins where the cell outline became indistinct and the nucleii appeared to lie in a gradually spreading syncytium. Many foci of the cytopathic effect were seen in the cell layer. The identity of the virus was confirmed in monkey kidney cells by

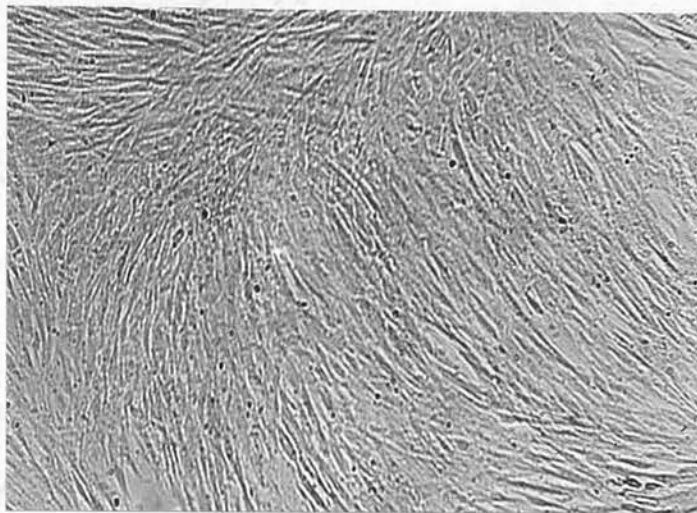
neutralization with known measles convalescent human sera.

#### Foetal lung cells

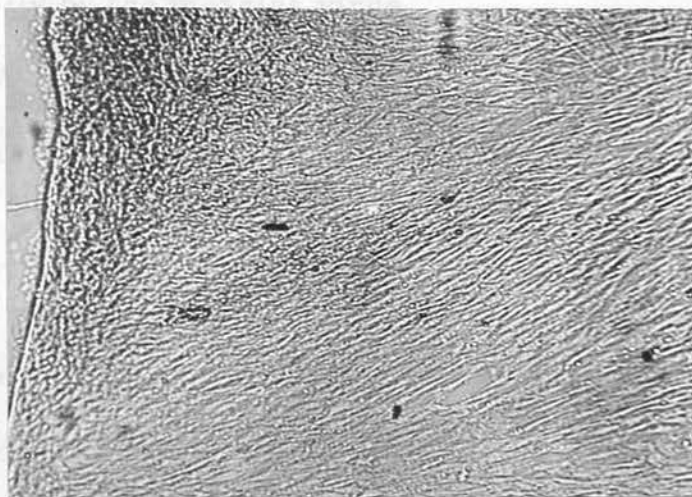
The source of these cells was the lungs obtained from 14 week old human foetus. The lungs were trypsinised, and the cells grown in Eagles basal medium supplemented with 10 per cent calf serum. The monolayers formed were split up every third or fourth day in an attempt to establish a cell-line, in the way described by Hayflick (1961). These cells were received in this laboratory in their eleventh passage from Dr. Sonndabend, who isolated them.

These foetal-lung cells were maintained for a further 18 passages, and were also stored frozen at  $-70^{\circ}$ . The method adapted to freeze these cells was the one described by Hayflick (1961).

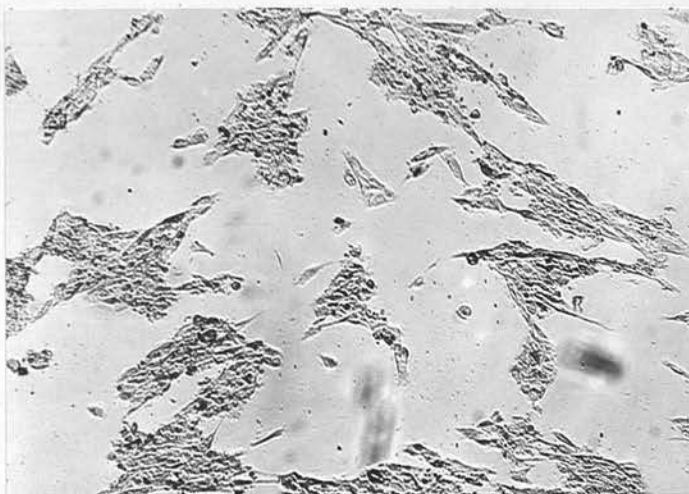
After the formation of the monolayer the growth medium was changed. The cell layers were washed with P. B. S. and inoculated with 1 ml. of HEP-2 grown measles virus, (titre  $10^{3.5}$  TCID<sub>50</sub>/ml.) and ferret kidney grown canine distemper virus (titre  $10^{3.0}$  TCID<sub>50</sub>/ml.) After allowing the inoculum to remain in contact for 2 hours at room temperature, the inocula were replaced with 1 ml. of maintenance medium (Eagles basal/medium with 3 per cent calf sera).



Human foetal lung cells (x80). Uninoculated controls.



Human foetal lung cells (x80). 72 hours after inoculation with canine distemper virus (wild strain 2) No discernible effect.



Human foetal cells (x80). 72 hours after inoculation with measles virus (HEP 2 adapted strain).

The cytopathic effect in all the measles inoculated tubes was seen within 5 to 8 days. This consisted of breaking up of the cell layer into small multinucleate syncytial areas (see Fig. on page.134.). Neutralisation of this effect by a known measles anti-serum (rabbit and human convalescent measles sera were used) indicated that it was caused specifically by the measles virus.

With the distemper virus inoculated tubes no change was noticed. On the 10th day after inoculation the cells were detached from the glass and, with the tissue culture fluid, were passaged to a further set of tubes. Five such passages were made and no cytopathic effect was seen in any. The tissue culture fluid from the 2nd and 4th passage was inoculated intra-nasally as well as intra-peritoneally to 2 ferrets which remained normal throughout the observation period of four weeks.



### Measles virus in dog kidney

Kidneys obtained from young puppies (2 days old) were processed and a monolayer was obtained. These were inoculated with the monkey kidney grown virus, as well as with stored acute blood, from which measles virus had been isolated in monkey kidney cells.

In the tubes inoculated with monkey kidney grown virus, the virus grew easily, and the first cytopathic effect was seen after 14 days in unstained preparations. This consisted of giant cells which were scattered throughout the cell monolayer. Many of the marginal cells showed the "stranding" already described.

In the tubes that were inoculated with stored acute blood, no C. P. E. was seen, even after three passages at ten day intervals.

Further work with the dog kidneys had to be stopped because it became impossible to obtain a regular supply of this tissue.

The following experiment was similar to that of Schwarz and Zitel (1959). One set of tubes (a) was maintained, by repeatedly changing the maintenance fluid, from another set (b). blind passages were made every 14th day.

Adaptation of the measles virus to grow in calf kidney cell  
monolayer.

Successful attempts to grow the measles virus in bovine kidney tissue culture were made by Schwarz and Zirbel (1959) but Warren and Cutchins (1957) failed. Hence, an attempt was made to see if the measles virus could be grown in bovine kidney cells.

Kidneys from 1 day old calves were collected from the slaughter house and processed as described for ferret kidney (page 81 ). When the cells had settled on the glass surface and were just spreading (5th day), the growth medium was poured off, the cells were washed once with P.B.S. and inoculated with  $\frac{1}{2}$  ml. of HEP-2 grown virus (titre  $10^{5.2}$  TCID<sub>50</sub>/ml.); this was allowed to remain in contact for 2 hours and replaced with growth medium and the tubes were then rolled. A monolayer was formed 12 days later. The growth medium was changed to maintenance medium, which was changed every 3rd or 4th day.

The following experiment was similar to that of Schwarz and Zirbel (1959). One set of tubes (a) was maintained, by repeatedly changing the maintenance fluid; from another set (b), blind passages were made every 14th day,

using 1 ml. of the pooled tissue culture fluid and the cells released by freezing and thawing.

In the 5 tubes (a) that were inoculated, and only the maintenance fluid was changed, giant cells similar to those seen in human or monkey kidneys were seen on the 43rd day and their nature was observed until the 60th day.

In the tubes (b) that were passaged, definite C. P. E. was seen in the 4th passage.

Passage Number	Cytopathic effect seen on
1	Nil
2	Nil
3	Few small giant cells on the 14th day.
4	Definite giant cells and syncytial formation by 12th day.

The identity of the virus in the fourth passage was checked by neutralisation with an anti-measles serum in calf kidney tissue cultures.

Thus, definite evidence of viral growth in calf kidney cells was obtained on the 43rd day; a period of 32 days shorter than that in Schwarz and Zirbel<sup>2</sup> experiments. This may be attributable to the use by these authors of much older monolayers of calf kidney.

In another series of exactly the same experiments the wild II strain of the canine distemper virus was introduced into calf kidney tissue cultures by the methods described above and a watch was kept for any C.P.E., throughout the whole time, however no effect was observed and it was concluded that the canine distemper virus did not grow in calf kidney cells.



SECTION IIIDistemper in dogs.

Dogs were brought to the out-patient clinic in the chronic stage of distemper for euthanasia. These were usually puppies less than a year old. A typical clinical picture of one of these dogs in brief is as follows. An eight month old collée pup became listless, with running nose, 'red' eyes, went off food; after about a week of this it settled down and appeared normal. However, the fever ( $105^{\circ}\text{F}$ ) started over again with vomiting and diarrhoea; a few rashes and vesicles were seen on the inner aspect of the thighs and belly. This fever alternated with short periods of normal temperature and behaviour. After four weeks of this type of intermittent temperature and bouts of gastro-enteritis, the puppy finally developed symptoms of distemper such as a continuous high-temperature ( $103.6^{\circ}\text{C}$  -  $106.4^{\circ}\text{F}$ ) with copious muco-purulent discharges from the eyes and nose. It was extremely wasted. During this period the dog was treated with full doses of penicillin, streptomycin and tetracycline.

The three seven-week old puppies all from one litter

from which strain III was isolated had a more acute course. All took ill with severe gastro-enteritis and when brought to the clinic about 12 days later they were extremely dehydrated, in fact two puppies were moribund.

One puppy, a six-month old cocker spaniel, was brought to the clinic on the 5th day of fever, as it was off food and had a running nose. It was provisionally diagnosed as an early case of distemper. Three ml. of venous blood was withdrawn. This was the source of strain I.

An attempt to grow the canine distemper virus in monkey kidney cells and in cells and cell lines of human origin.

Monkey kidney: 5-day old secondary monkey kidney cell monolayers, after careful selection, were used. Virus source was the "wild strain" I isolated and grown in ferret kidney cell monolayer. (Titre was  $10^{3.5}$  TCID<sub>50</sub>/ml.).

The experiment conducted is given below in a diagrammatic manner.

1. 5 day old Mk. T. C.  
Inoculated with virus - observed for 14 days  
No C. P. E.  
Harvest TCF  
(Cells + TCF)  
passaged to

2. 7 day old Mk. T. C. - observed for 18 days  
No C. P. E.  
Passage Harvest (cells + TCF)  
Passage to 2 ferrets.

3. 7 day old Mk. T. C. - Observed for 18 days  
No C. P. E.  
Passage Harvest (cells + TCF)
4. 7 day old Mk. T. C. - observed for 21 days.  
No C. P. E.

Distemper virus in ferret kidney cell monolayer

Ferret kidneys were processed as described under Methods and Materials. Good monolayers were selected and inoculated with whole blood, drawn from ferrets in the acute stage of distemper. 1 ml. of whole blood was inoculated into each of the four tubes, and was allowed to remain in contact with the cell layer for two hours at room temperature. The tubes were then washed free of all blood cells with P.B.S. and replaced with maintenance medium. The composition of the maintenance medium was as follows:

Eagle medium x 10.    ..    ..    ..    10 ml.

Earle's S.S. containing  
0.5% Lactalbumin hydrolysate    ..    90 ml.

To 92 ml. of the above mixture was added 5 ml. of 4.4 per cent soda bicarb., 3 ml. of calf serum and 1 ml. of antibiotic mixture to give a final concentration of 100 units of Penicillin and 100 ug of Streptomycin. The inoculated tubes were incubated at 37°C and were observed daily.

The maintenance medium was changed every third or fourth day. After 18 days, (in scattered areas, in 3 out of 4 tubes, cells showed a granular appearance with fine vacuolations). In another two or three days small giant cells with 4 to 12 nucleii could be seen. By the 23rd day, all the

four tubes were showing definite C. P. E. The cells at the margins of cell layer were showing bizarre shapes with "stranding" of cells or "The spider" cells.

The tissue culture fluids were harvested on the 30th day when most of the cell surface was involved. The C. P. E. seen was in small patchy areas, unlike the large areas of syncytia seen in measles. The pooled harvest was centrifuged for 5 minutes at 1500 R. P. M. and 0.2 ml. of the supernatant inoculated into 15 more ferret kidney monolayers and the tubes were incubated at 37°C. The C. P. E. was seen for the first time after 7 days, and all the tubes were involved by the 10th day. The tissue culture fluids from all the tubes showing good cytopathic effect were harvested and pooled, every two days, and replaced with fresh maintenance medium. The cells in some of the tubes remained up to 42 days. The pooled harvests were stored at -70°C.

The identity of the virus was proved by neutralisation tests when 0.5 ml. quantities of the virus and known high titre distemper anti-sera (B. W. & Co)., were mixed and incubated for 1 hour before inoculation, into ferret kidney tissue culture, and into ferrets.

5 ml. of the virus was inoculated into Roux bottles



containing ferret kidney monolayer and the virus stock was built up.

#### Titration of the virus

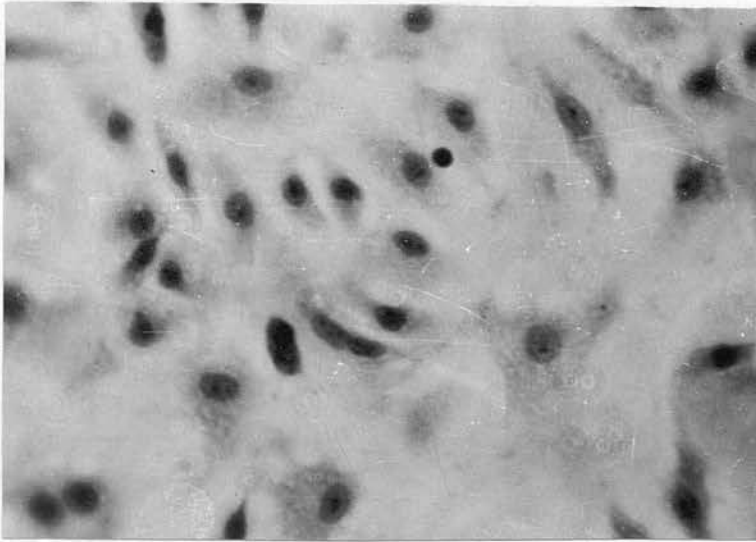
The pooled harvest from Roux bottles was given a clarification spin and the supernatant was titrated in Ferret kidney cell monolayer.

The titre of this pool was  $10^{3.5}$  TCID<sub>50</sub>/ml.

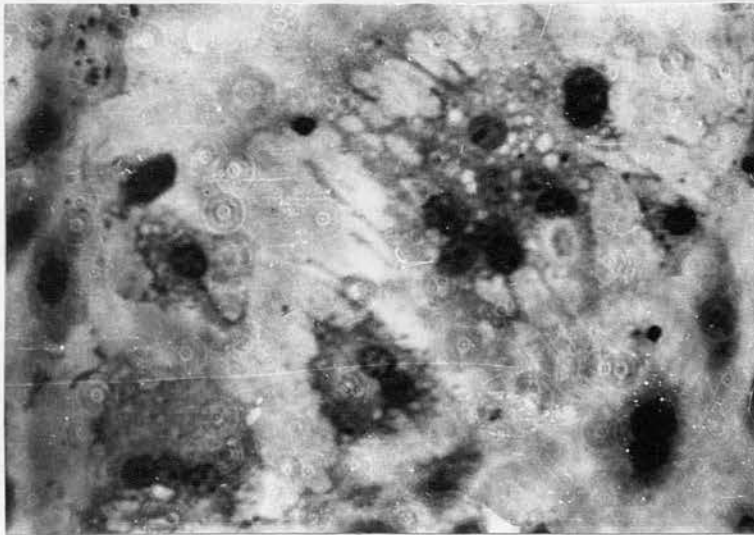
#### Incubation of the virus at different temperatures

As the normal temperature of the natural host of distemper, namely 'dogs', is 101.8°F. for acute dogs, (Paterson, 1957) and that of the susceptible animal, the ferret, is 101.6°C (UFAW - 1957), the following experiment was done to find out if there was any appreciable difference that could be observed by incubating the inoculated tubes at 39°C.

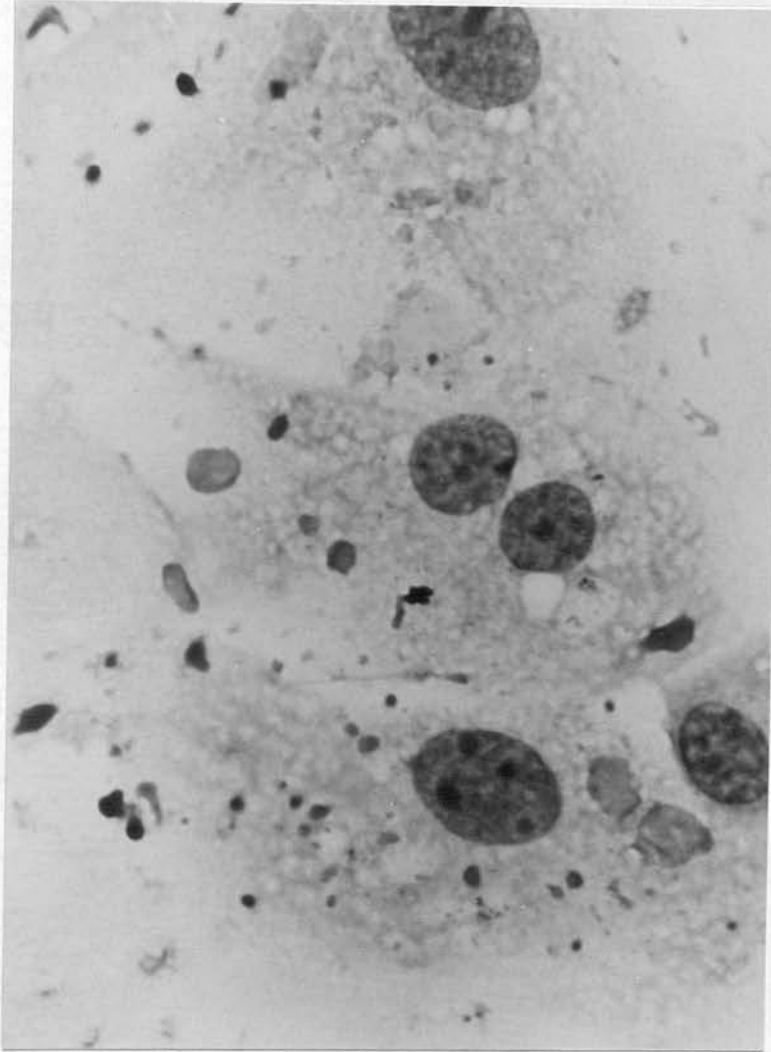
Eight tubes of ferret kidney cell monolayer with coverslips were inoculated with 0.2 ml. of distemper virus containing tissue culture fluid ( $10^{3.5}$  TCID<sub>50</sub>/ml.). Four of these tubes, together with four uninoculated controls, were incubated at 39°C. The other four, together with four more controls were incubated as usual at 37°C. In the tube incubated at 39°C the C. P. E. was seen as early as 48 hours and 2 to 8 nucleii could be seen in each affected cell. The



Uninoculated Ferret kidney cell monolayer,  
15 days incubation at 37°C. Giemsa Stain x40.

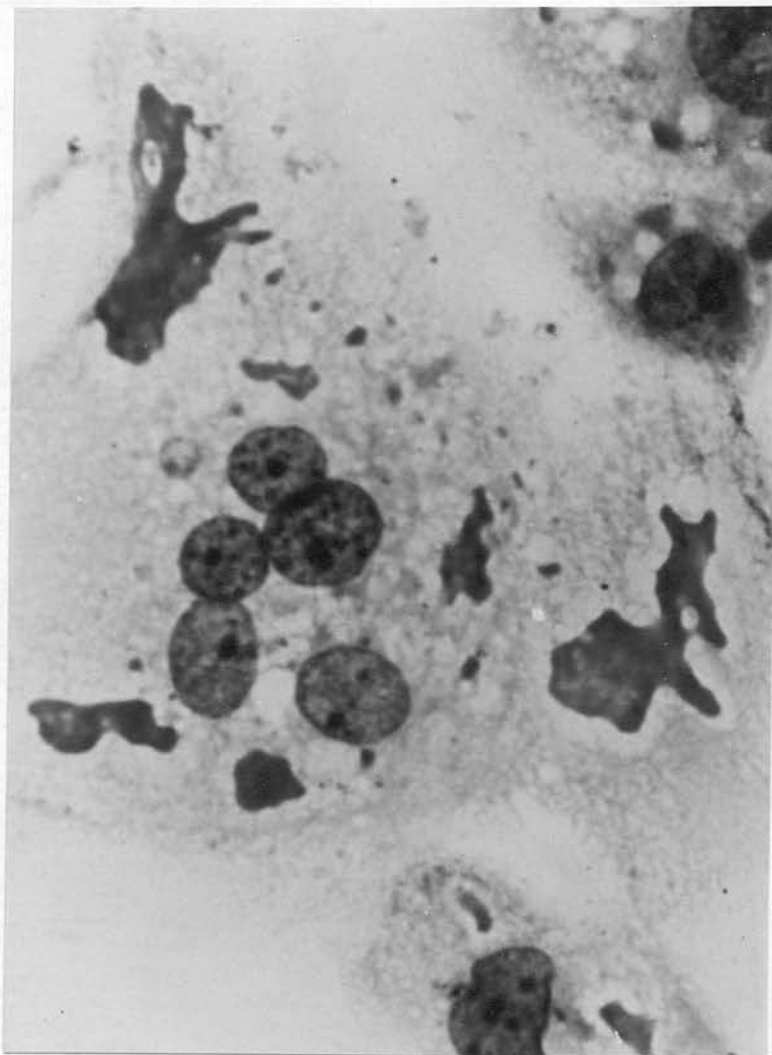


Ferret kidney monolayer inoculated with wild  
strain II 8 days after inoculation and incubated  
at 37°C. x40.  
Giemsa Strain.                      Coverslip preparation.



Ferret kidney cell monolayer inoculated with Wild Strain II. 4 days after incubation at 37°C. Note the number of nuclei and the size and distribution of the inclusion bodies, as compared with a similar preparation but incubated at 39°C.

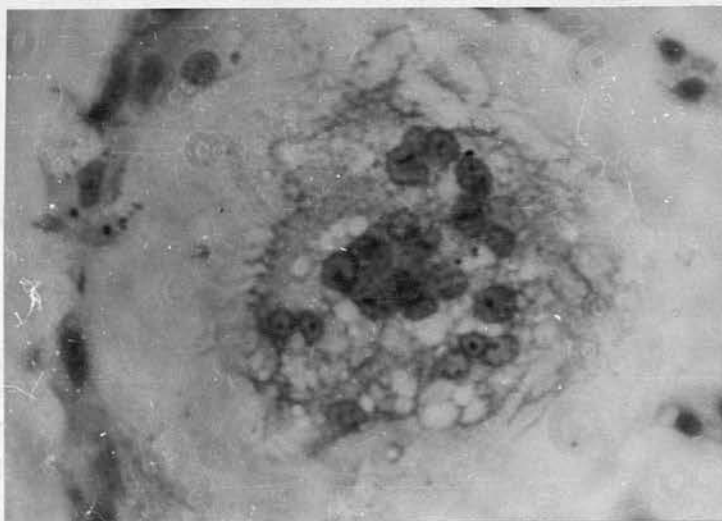
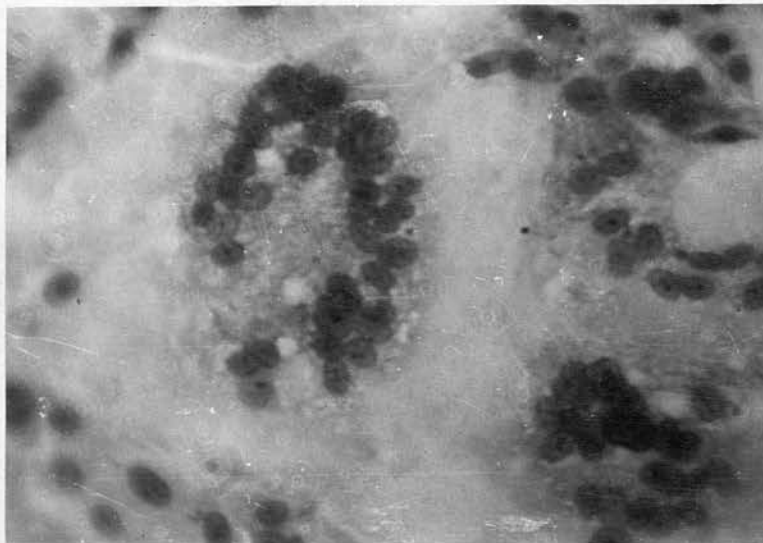
Giemsa Stain      x 240.



Ferret kidney cell monolayer: another preparation similar to the previous one, but incubated for 8 days at 37°C.

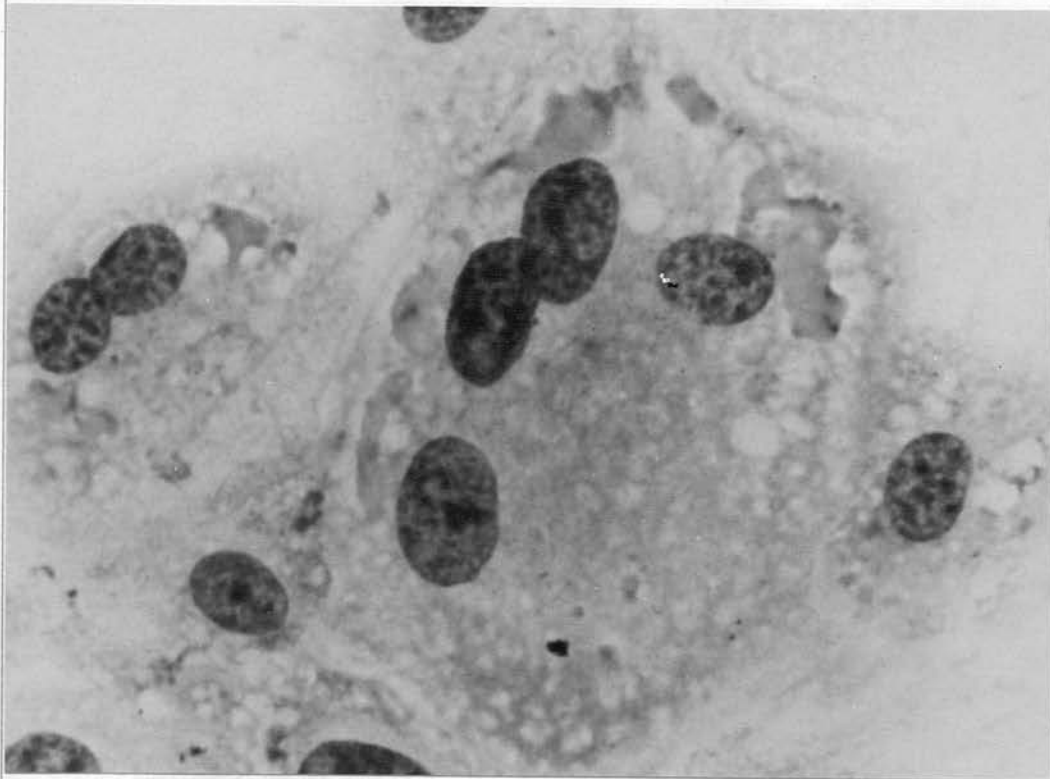
Giemsa Stain x 240.





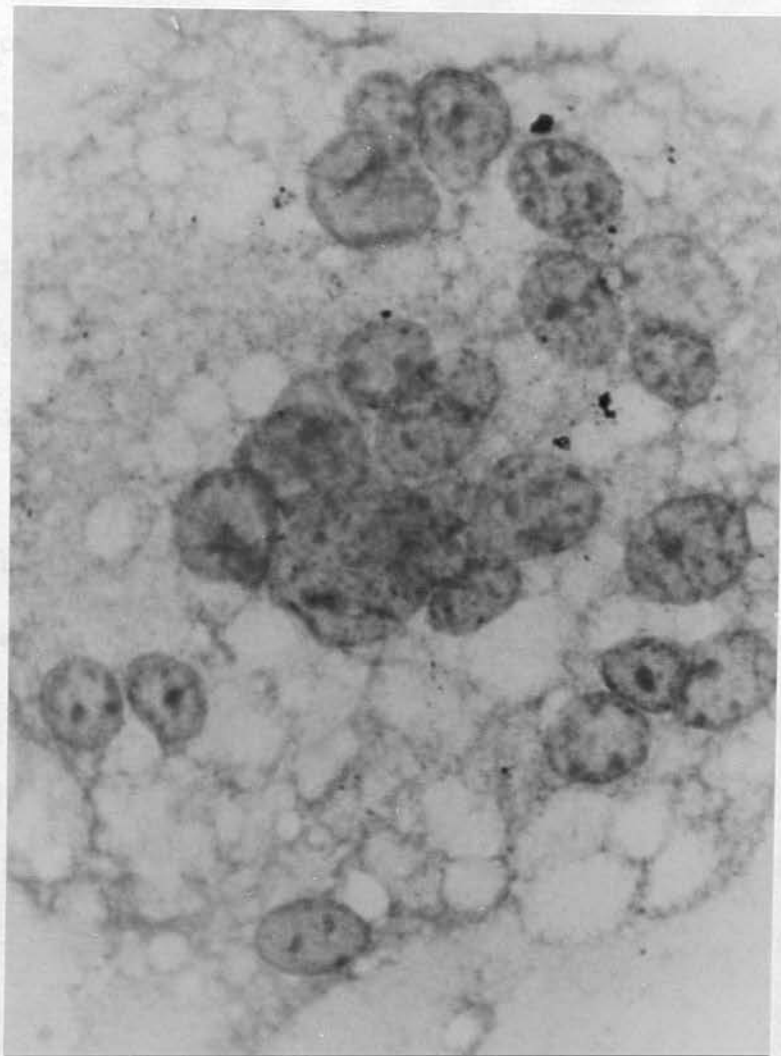
Ferret kidney monolayers inoculated with Wild  
Stain II, and incubated at 39°C. for 8 days.  
Giemas Stain x40.

Note the larger giant cells over the great number  
of nuclei, arranged ptheripally. Coverslip  
preparation.



Ferret kidney cell monolayer inoculated with Wild Strain II 4 days after inoculation at 39°C. Note the peripheral arrangement of the nucleii and the intra-cytoplasmic inclusion bodies.

Giemsa Stain      x 240.



Ferret kidney cell monolayers inoculated with Wild Strain II. Same preparation as on page at a higher magnification of the lower picture

Giemsa Stain x 240.

cytoplasm showed large areas of acidophilic inclusion bodies, arranged peripherally to the nucleus. There were fine vacuolations of the cytoplasm with scattered minute inclusion bodies. In the tubes ~~un~~inoculated at 37°C these changes were seen at least 48 hours later. Even then the "giant" cells had only 2 to 3 nucleii and the inclusion bodies were very much smaller and scattered throughout the cytoplasm. (See photograph no. 145-7).

In the tubes incubated at 39°C large giant cells with several nucleii varying from about 25 to 150, and even in excess of this, were seen. These nucleii appeared to have a definite arrangement and were seen regularly placed at the periphery of the cell, with or without a central cluster. (See photograph no. 146-150). The cytoplasm was highly vacuolated, and the cytoplasmic inclusion bodies were absent; probably they had been released into the tissue culture fluid and the vacuolation represented the empty spaces left in the process.

In the tube inoculated at 37°C the changes seen were slow to appear; on the 8th day, small giant cells with up to a dozen, or more, nucleii were seen. The intracytoplasmic inclusion bodies were seen placed peripherally to the nucleii and were irregular in shape. Smaller inclusion



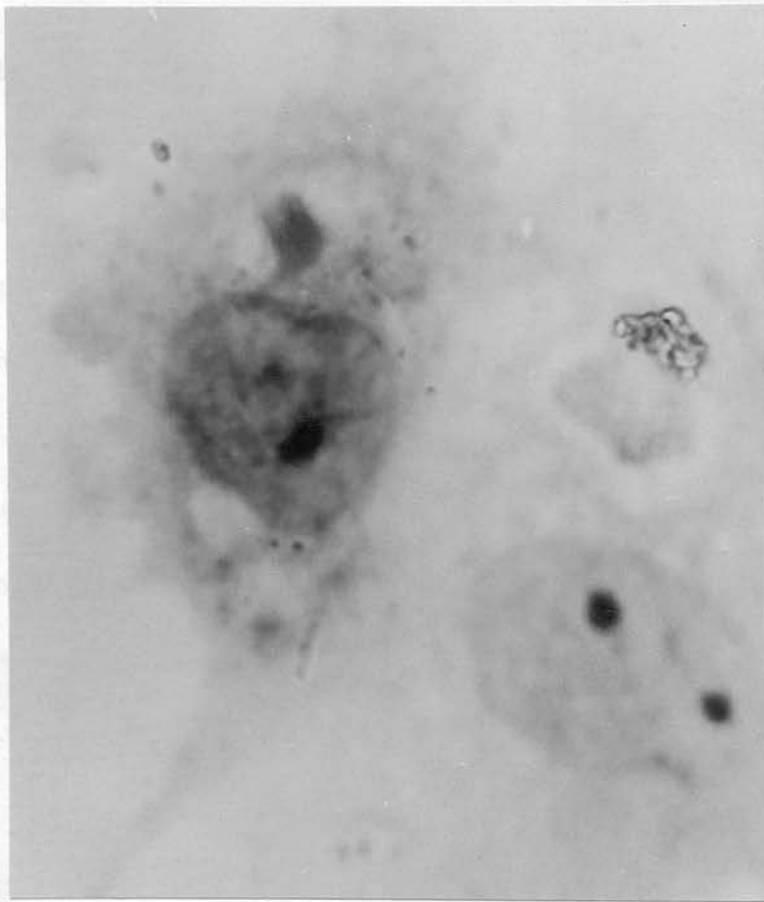
bodies were also found scattered through the cytoplasm.



Ferret kidney monolayer infected with canine distemper virus. (Wild Strain II).

Incubated for 8 days at 37°C. x800. Phloxine-tartrazine stain.

This preparation shows several intracytoplasmic phloxinophilic inclusion bodies of various sizes. Note the granular appearance of the inclusion bodies.



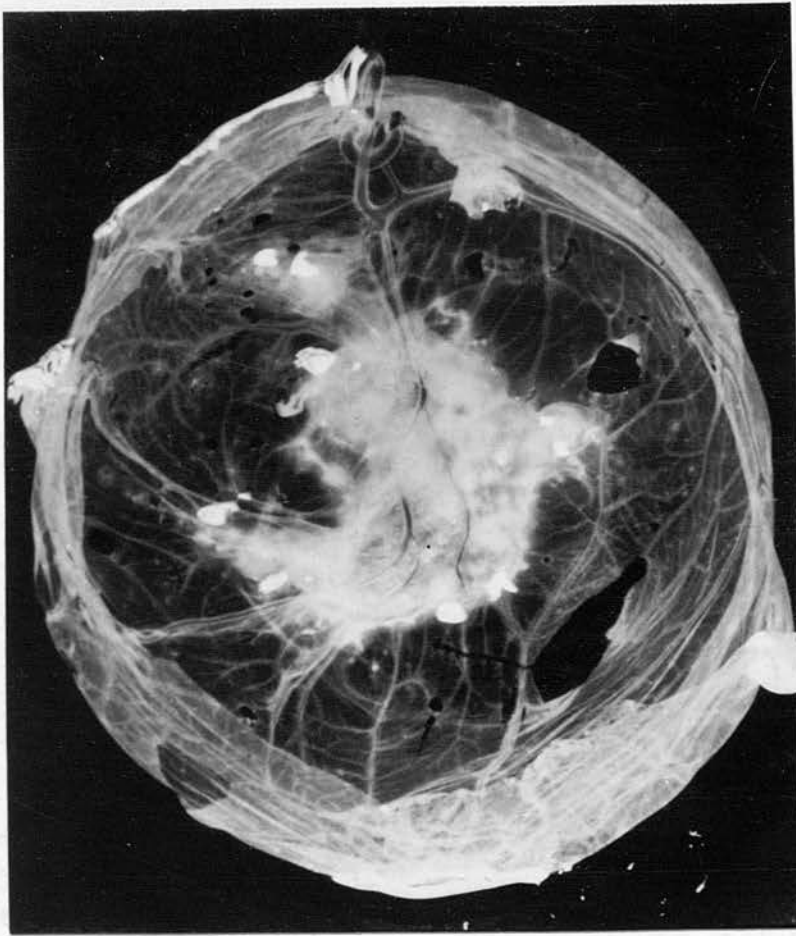
Ferret kidney monolayer infected with canine distemper virus. Another field from the same preparation. This may be an earlier stage than the previous one. Note the indistinct outlines of the diffuse intranuclear inclusions and the cytoplasmic inclusions adjacent to the nucleus.

Distemper in eggs When the Epivax egg adapted strain of the distemper virus is seeded on to the chorio-allantoic membrane of the chick embryo obvious pock-like lesions are produced but no lesion results when wild unadapted strains are used.

It is thus possible to propagate the Epivax strain in eggs and to obtain large yields of the virus for laboratory methods; furthermore the method can be adapted for use in titrating the virus and also it can be used in neutralisation tests.

A simple technique was used by which the large numbers of eggs used in these procedures could be handled rapidly. Fertile eggs 6 - 7 days old were opened by removing the shell over the air sac and a drop of the inoculum was placed on the shell membrane. Subsequent gentle laceration of the shell membrane enabled the inoculum to seep through and to spread to a localised area on the underlying chorio-allantoic membrane. The earliest pocks were detected after 4 - 5 days' incubation at 37°C and fully developed lesions were present by 6 - 7th day (See coloured photograph). The speed with which the inoculations can be made and the ease with which the infected membranes can be harvested made this





Canine distemper virus on Chorio-allantoic membrane

Canine distemper virus - 7 days' growth on the chorio-allantoic membrane of hen's egg. The inoculation was done via the air sac.

technique more valuable than the conventional method of Beveridge and Burnet (1946).

This technique was used to estimate the infectivity titre of viral antigens to be used in neutralisation tests to be described later. An example of one such titration is given below.

#### Titration of virus in eggs

Serial ten-fold dilution of virus in 199.

Five eggs for each dilution and 0.1ml.  
per egg.

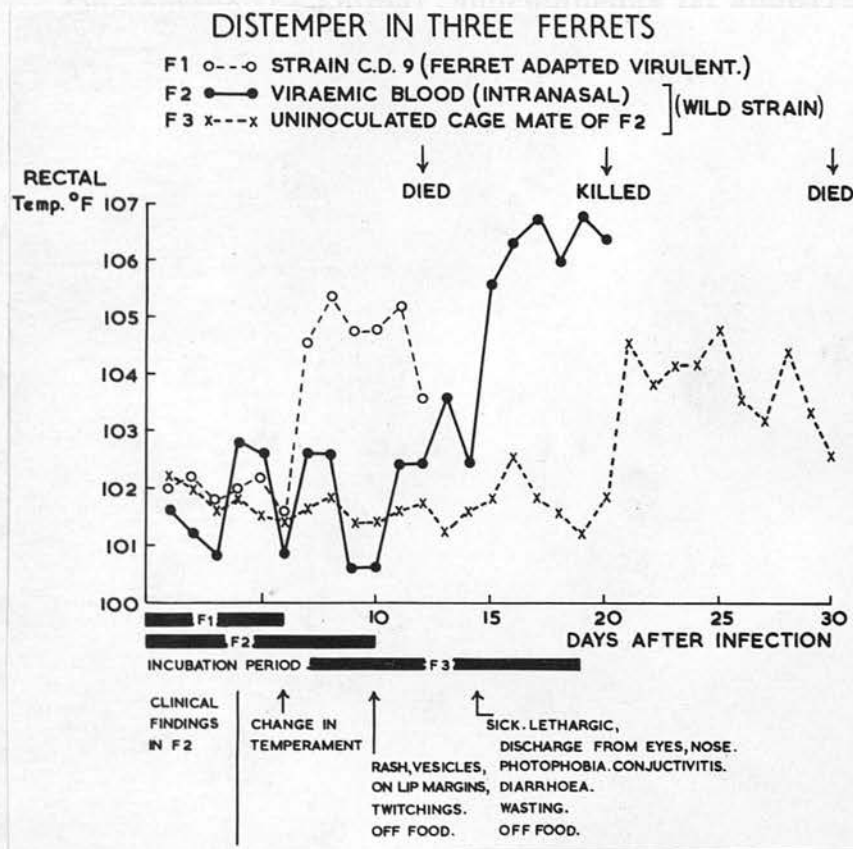
Dil. of virus	$10^0$	$10^{-1}$	$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$	$10^{-6}$
No. of eggs showing lesions	5	5	5	4	1	0	0
	/	/	/	/	/	/	/
No. of eggs inoculated	5	5	5	5	5	5	5

titre of the virus is  $10^{-3.5}$  EID 50/0.1 ml.  
(Reed - Meunch 1938)

Post mortem examinations were carried out on the uninoculated ferret, (3) that died of distemper. (See temperature chart <sup>on</sup>, page 158. ) and also on the control uninfected litter mate.

#### The findings were:

The animal appeared wasted, thin and had 'staring'



fur. The eyelids were glued together with a yellowish sticky purulent discharge. The nose was dry, fine rashes and minute vesicles were seen at the angle of the mouth and around the anus. The pads appeared somewhat thickened and red.

On opening the animal subcutaneous fat appeared deficient and there was mild generalised congestion. Inguinal, axillary, submental and cervical lymph glands were prominent but they appeared pale and not congested.

Abdomen There was no excess of peritoneal fluid. The viscera, except for the liver, appeared congested. Fine petechrial haemorrhages were seen on the mucosa of the intestines which contained malaenic material. The spleen was enlarged to about one and a half times the usual size and was intensely congested. The liver appeared slightly oedematous but normal in colour. The bladder was distended with urine. The urinary deposit when stained with giemsa showed intra-cytoplasmic acidophilic inclusion bodies in the epithelial cells. The kidneys appeared congested but without the perinephritic fat. Suprarenals appeared normal. Mesenteric lymph glands were prominent.

Chest The lower lobes of both lungs showed congestion and there were scattered patchy areas of broncho-



pneumonia. The trachea and large bronchii contained frothy fluid and the mucous membrane was congested. The heart appeared normal.

Skull The meninges appeared congested as did the brain surface. There appeared to be excess of cerebrospinal fluid.

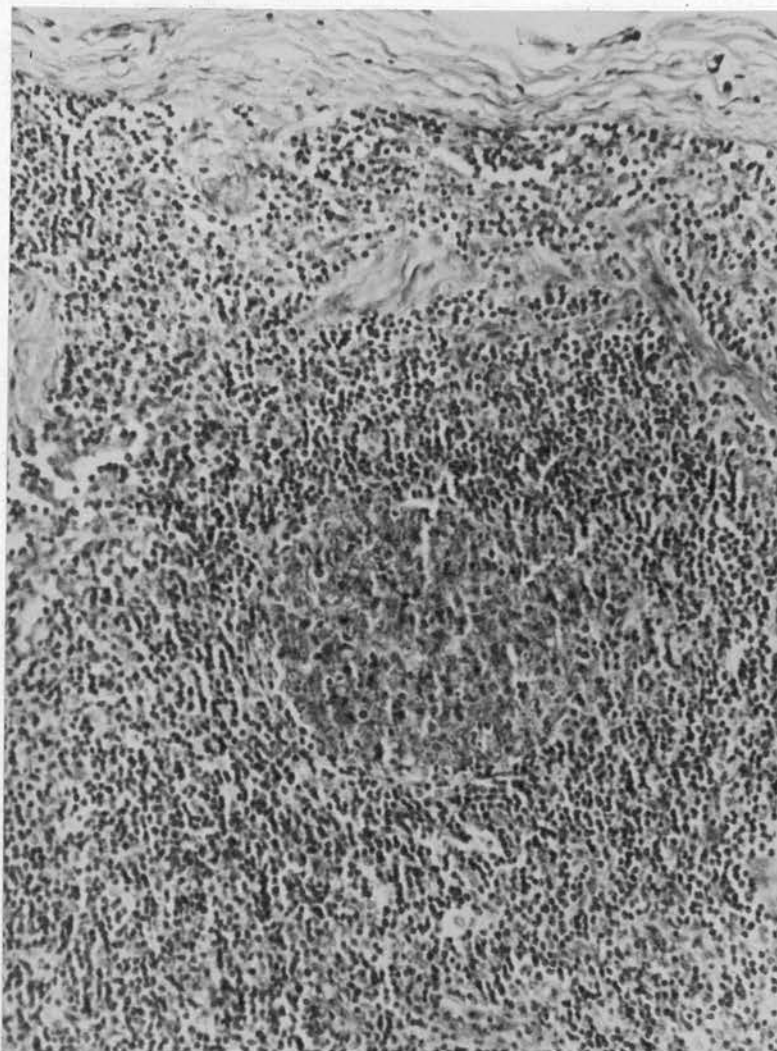
### Histopathology

Tissues removed at autopsy from the distemper infected and control ferrets were fixed in corrosive formal and sections were made and stained with haematoxylin and eosin.

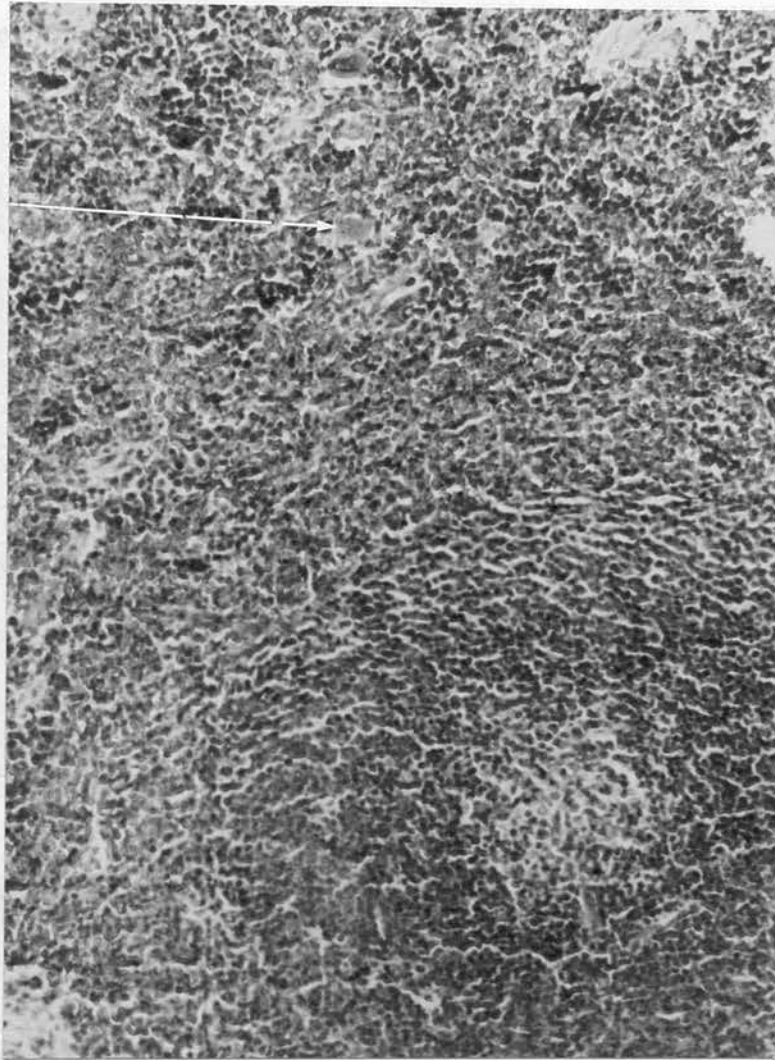
Relatively little abnormality was seen in the sections of brain, meninges, lung and intestine; no giant cells were found and slight congestion of the capillaries and a few minute haemorrhages were the only departures from normal.

The most obvious changes were those in the lymphoid tissues particularly in the lymph glands themselves, the tonsils and in the spleen; definite thickening of the bladder epithelium was also present. In the lymphoid tissues of the distemper infected ferret marked lymphocytic proliferation and extreme hyperaemia were striking features and many multinucleate giant cells resembling very closely

the Warthin-Finkeldey cells described in measles infected tissues were present. In the hyperplastic epithelium of the bladder inclusion bodies were also found in large numbers and in this situation appeared rather large numbers. In the following pages colour photographs contrast the normal histological appearance of ferret tissues with those of the infected animal.



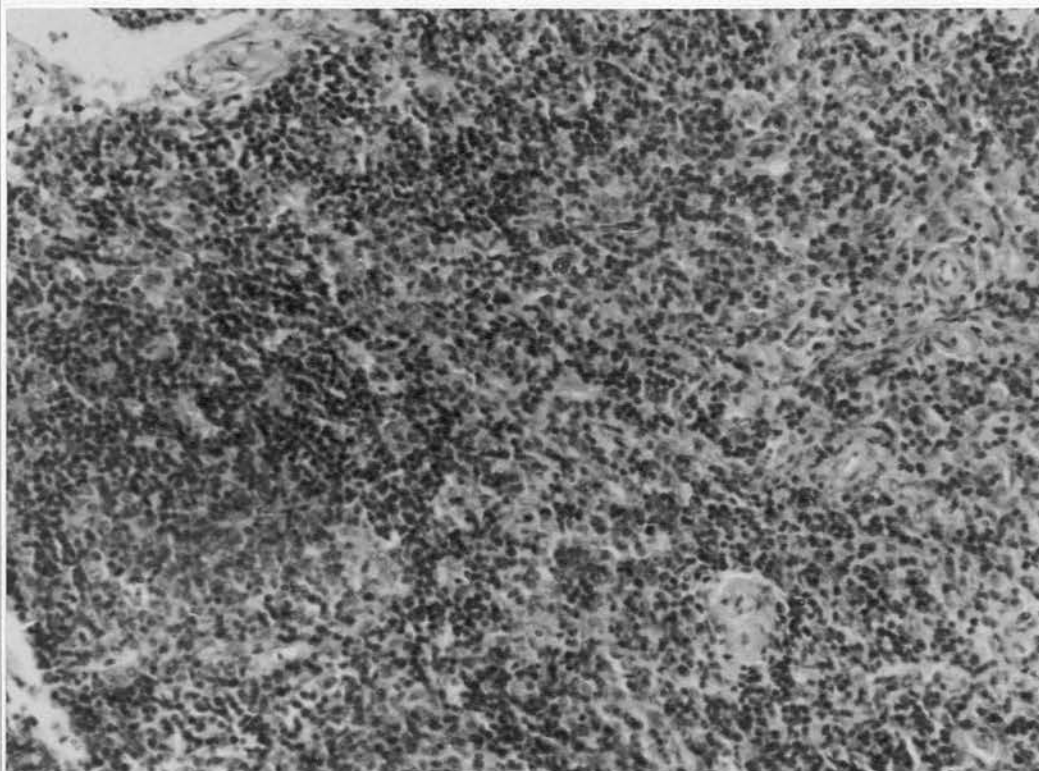
Lymph gland of a normal ferret. Haematoxylin and eosin  $\times 80$ . A normal germinal centre approximately 0.5 mm in diameter is seen surrounded by masses of small lymphocytes. Small numbers of macrophages are scattered throughout and occasional red blood cells are seen in the sinuses. The fibrous capsule is not thickened.



Lymph gland of a distemper infected ferret (the same animal). Haematoxylin and eosin x 80.

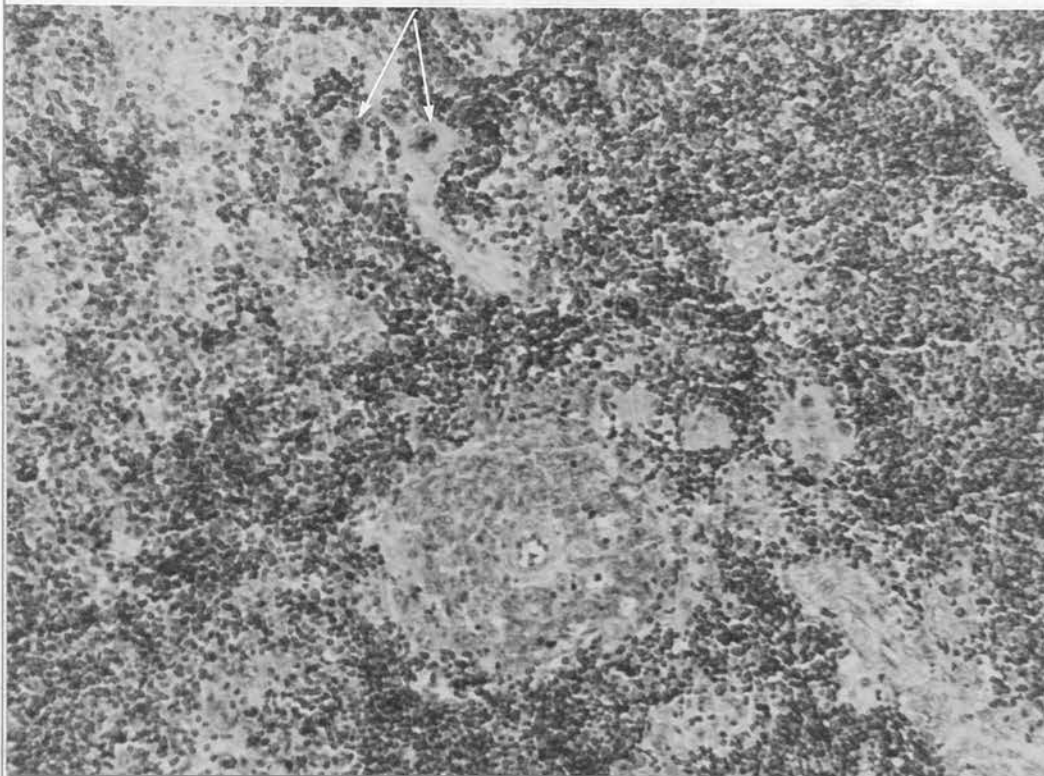
An enlarged germinal centre approximately 1 mm. in diameter and about double the size of the normal is seen to be the site of marked lymphocytic proliferation. Note the extreme hyperaemia throughout the whole gland and the distension of the sinuses with red blood cells. Multinucleate giant cells are seen distributed between the germinal centres and there is an increased number of macrophages.





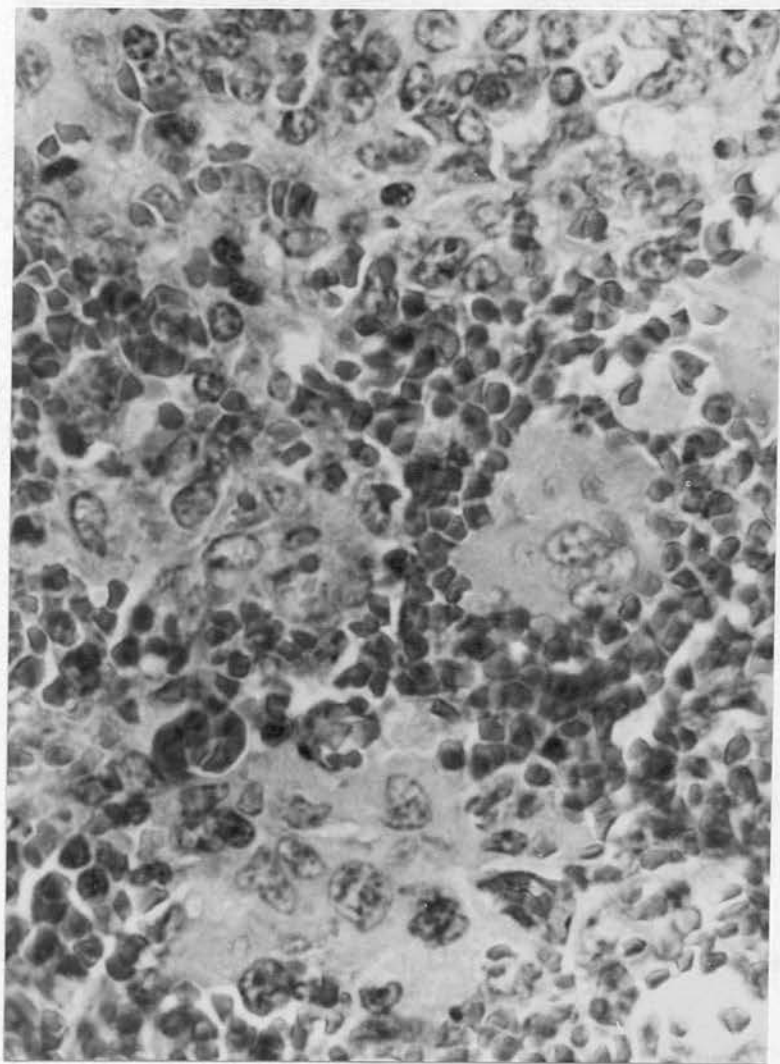
Spleen of a normal ferret. Haematoxylin and eosin x80.

The section shows a normal balance between the white and red pulps. A Malpighian corpuscle is seen to the left and is surrounded by the sinuses of the red pulp. Small numbers of macrophages are seen throughout.



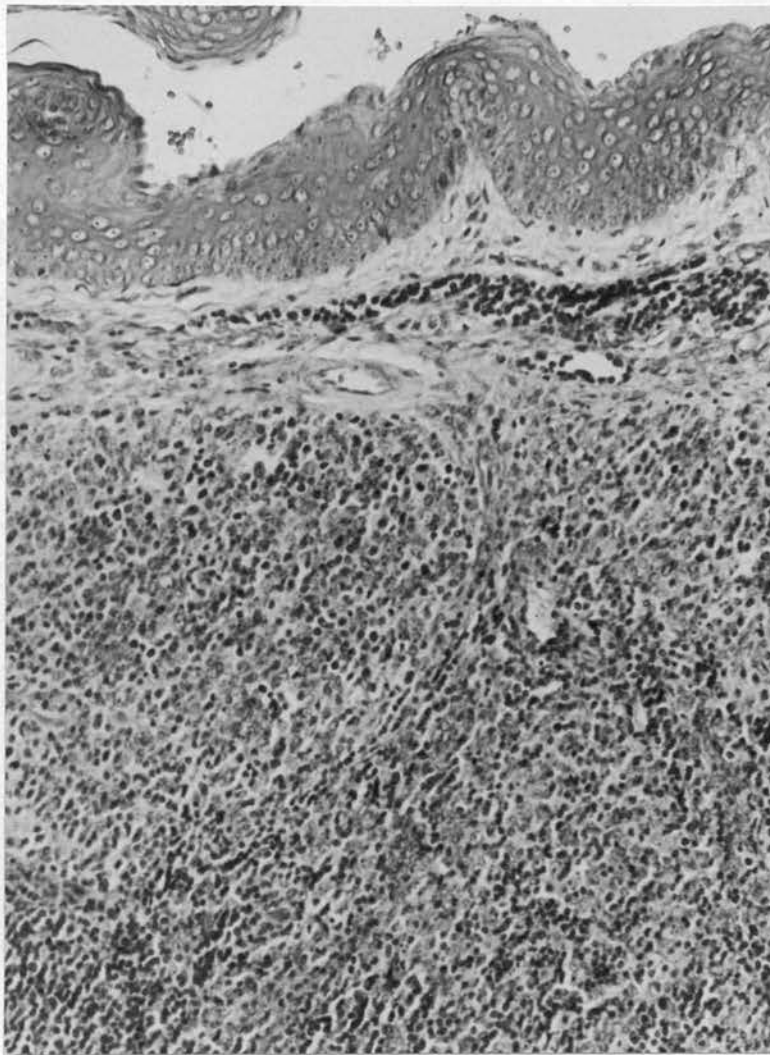
Spleen of a distemper infected ferret (the same animal).  
Haematoxylin and eosin x 80.

The section shows marked hyperaemia of the whole tissue. The sinuses are engorged with red blood cells and the red pulp is greatly increased in amount. Note the large numbers of multinucleate giant cells scattered throughout the red pulp.



Spleen of a distemper infected ferret. Haematoxylin and eosin x 240.

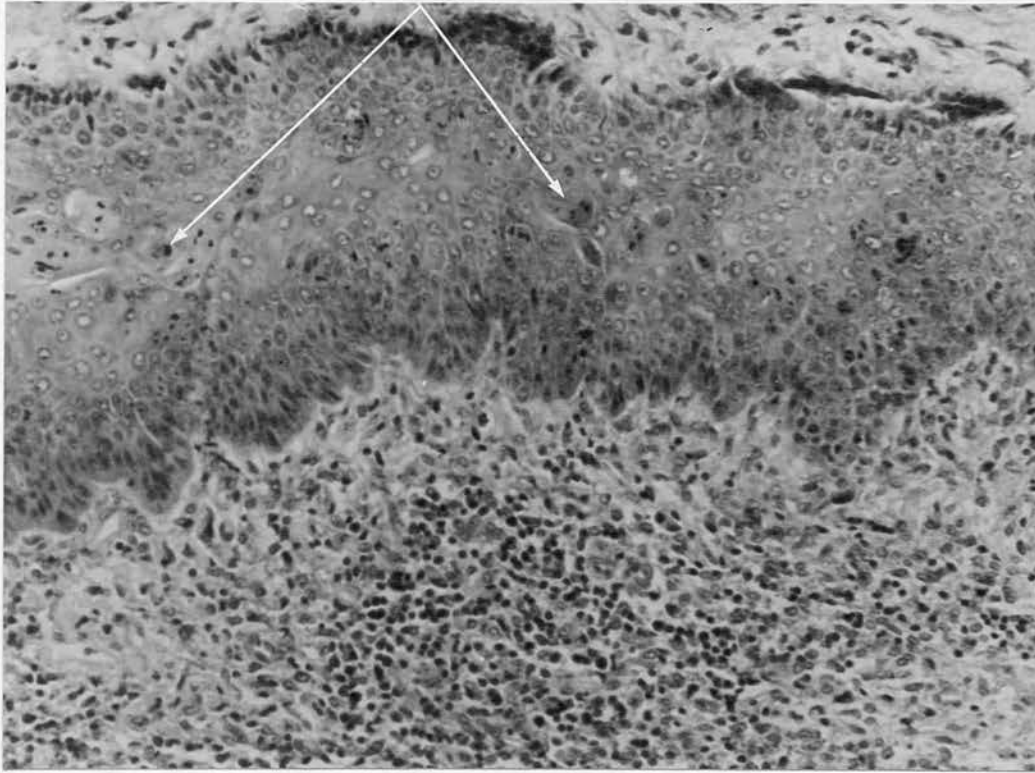
A higher magnification of the previous section to show the details of the structure of the multinucleate giant cells.



Tonsil of a normal ferret. Haematoxylin and eosin x 80.

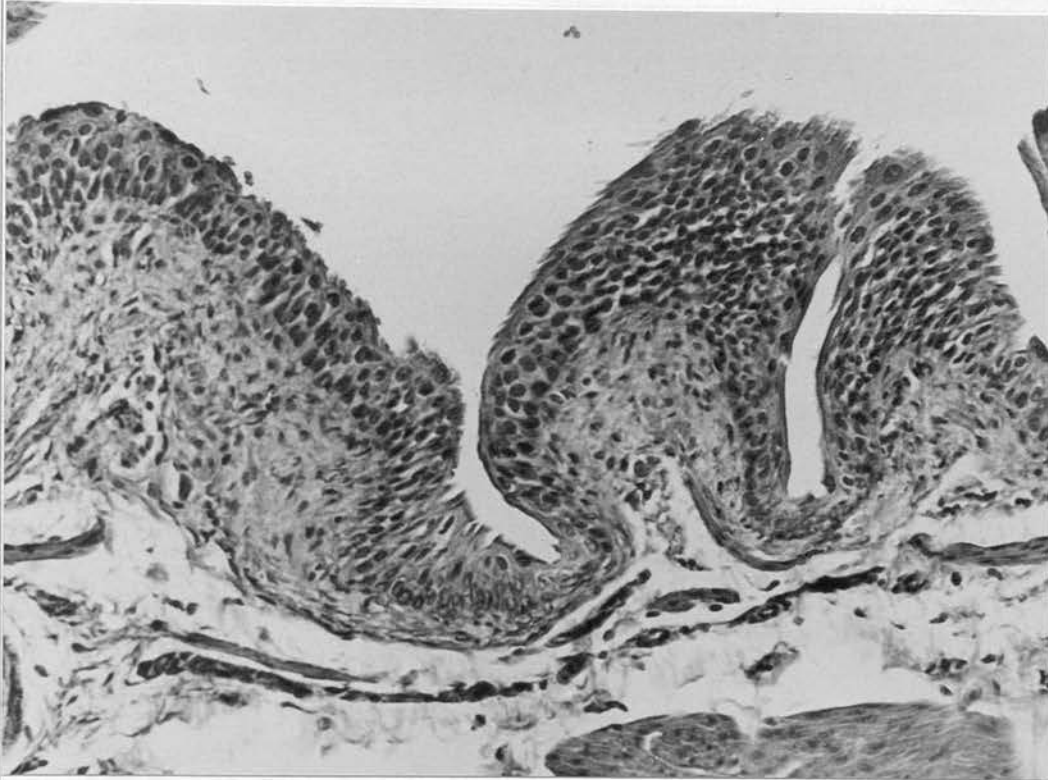
Normal lymphoid tissue is covered by a layer of stratified squamous epithelium 5 - 8 cells thick.





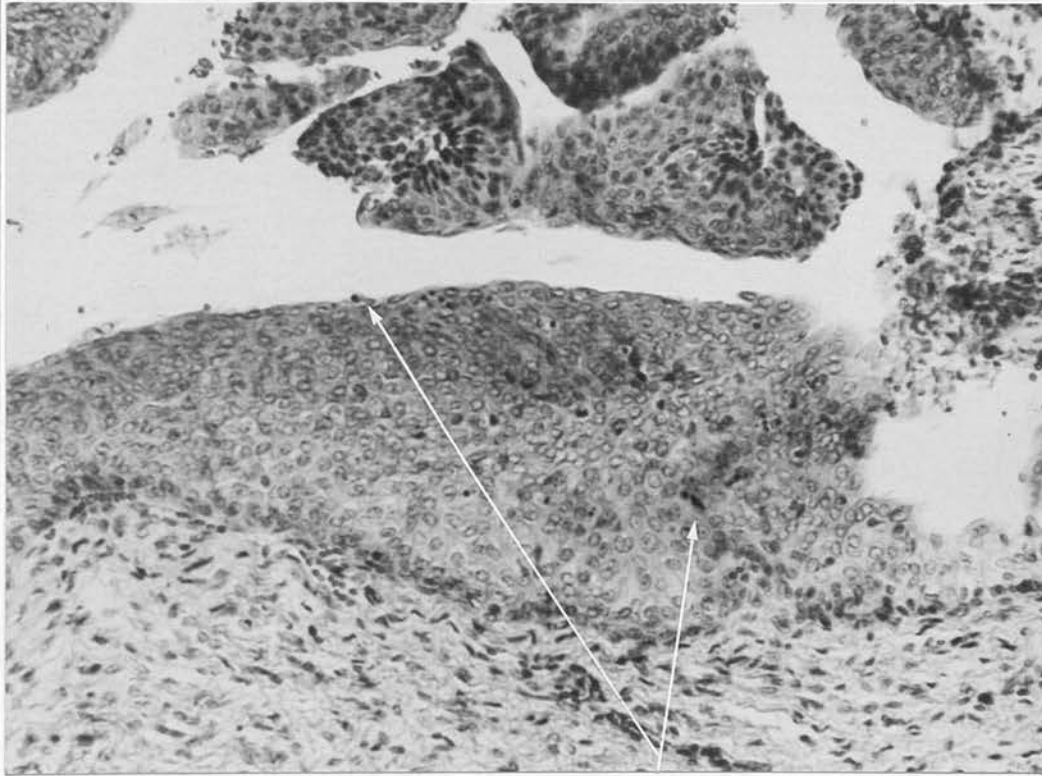
Tonsil of a distemper infected ferret. Haematoxylin and eosin x 80. This animal was killed when moribund 21 days after the intranasal instillation of the Wild I strain of the canine distemper virus.

The surface of the epithelium is covered by a layer of fibrinous exudate containing inflammatory cells and debris. The underlying stratified squamous epithelium shows marked hyperplasia and thickening to double the width of that in the normal. Many multinucleate giant cells bearing intracytoplasmic eosinophilic inclusions are seen scattered throughout this layer. The underlying lymphoid tissue is congested.



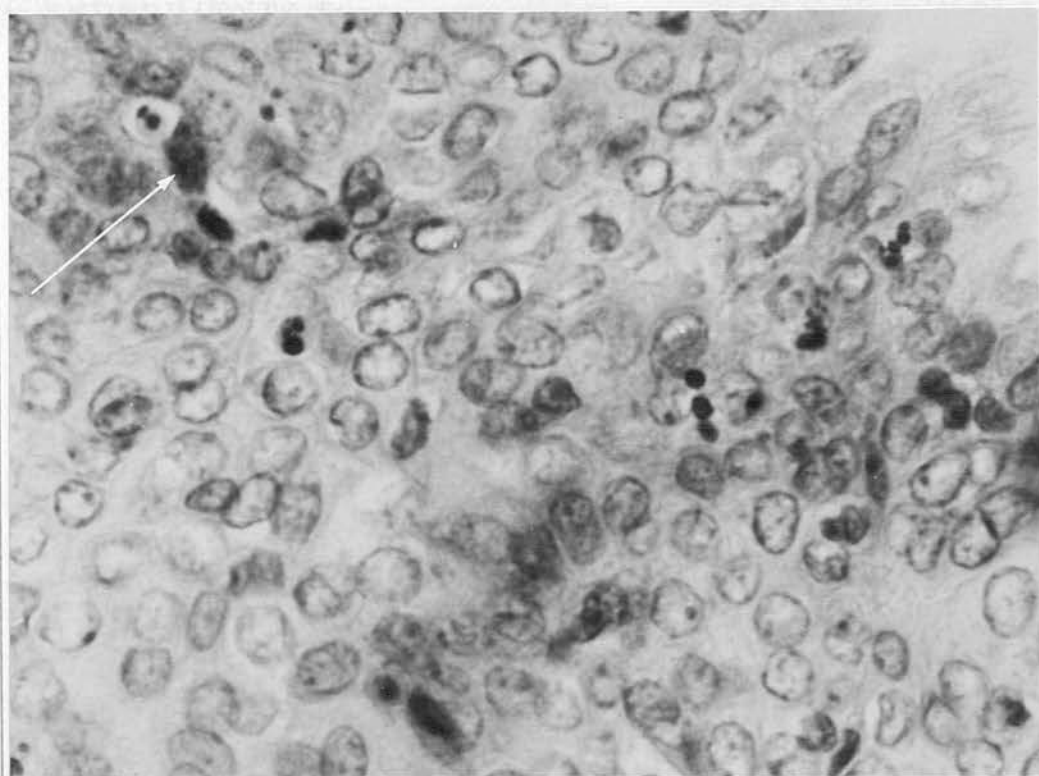
Bladder of a normal ferret. Haematoxylin and eosin x80.

The layer of transitional epithelium is 6 - 8 cells thick and occasional lymphocytes can be seen between the cells.



Bladder of a distemper infected ferret. Haematoxylin and eosin x 80.

The transitional epithelial layer shows marked hyperplasia and is approximately twice as thick as that seen in the normal control animal. There is infiltration with darkly staining mononuclear cells which appear to be migrating through the thickness of the epithelium. In some areas (e.g. to the right of the section) the bladder wall has been denuded of the inflamed epithelium.



A higher magnification photograph from the same section to show the infiltrating mononuclear cells and the acidiphilic inclusion bodies.



SECTION IVEXPERIMENTS TO STUDY THE EFFECT OF THE MEASLESVIRUS ON FERRETS

At the beginning of this work 3 pairs of breeding ferrets were obtained from the Royal Dick School of Veterinary Studies and from them a small colony of ferrets was developed which was known to be clean and free from inter-current infection. Most of the animals became perfectly tame and could be handled without gloves.

Adult ferrets      Eight adult ferrets 8 - 12 months old and weighing 950 - 1200 grams were selected for the first experiment. Each animal was anaesthetised with intra-peritoneal Nembutal before inoculation. The measles virus was the HEP 2 adapted strain (titre  $10.5 \text{ TCID}_{50} / \text{ml}$ )., and each animal received 0.5 ml. by intra-nasal instillation as well as 2.0 ml. intra-peritoneally.

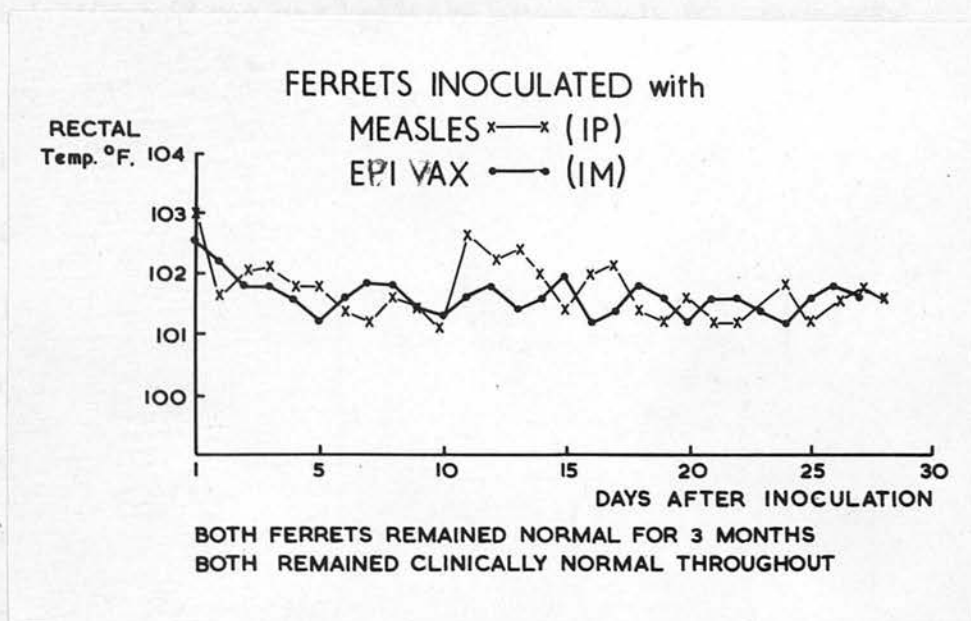
These ferrets were observed for four weeks; none showed any reaction or signs of illness. They appeared perfectly normal and put on <sup>weight.</sup> ~~which~~ paralleled with the controls. The temperature chart of one of these ferrets, along with another inoculated with Epivax is shown.

Rousell (5 mg per X. G.).

As control ferret from the same litter.

Control in the same litter, but received only 0.2 ml. of

T. C. F. from which 0.2 ml. control (1/2 X. G.)



It appeared normal.

At the post-mortem, the lungs appeared

Some of these ferrets were later used for the production of measles antisera, and for cross-protection tests.

#### Young ferrets

Three young ferrets, four weeks old (weight 100 to 150 gms.), were given intra-cerebral injection of 0.2 ml. of the same measles virus used in the above experiment and an intra-peritoneal injection of Cortisone acetate (Cortisyl.

Roussel) (2 mg per K. G.).

A control ferret from the same litter was also given Cortisyl in the same dosage but received only 0.2 ml. of T. C. F. from uninoculated control HEP.2. cells. Cortisone injections (2 mg per kg) were given daily for one week.

The experimental ferrets reacted by showing a rise in temperature. Two died after fourteen days and the third which ran a temperature (rectal) of 106., 106.2, 106.6, and 106.2°F, on the 14th, 15th, 16th and 17th days, was sacrificed on the 18th day. (The control animal remained normal during this period of observation). There was no diarrhoea, no rashes; no nasal or conjunctival discharges were evident and, apart from the reddening of lips and the temperature, it appeared normal.

At the post mortem, the brain appeared to be moderately congested and oedematous, and the spleen appeared normal in size. In the histopathological examination of sections no abnormality could be made out.

#### Suckling ferrets

A litter of eight 48-hour old ferrets was used in the following experiments.

Pair 1. Two animals were used as uninoculated controls.

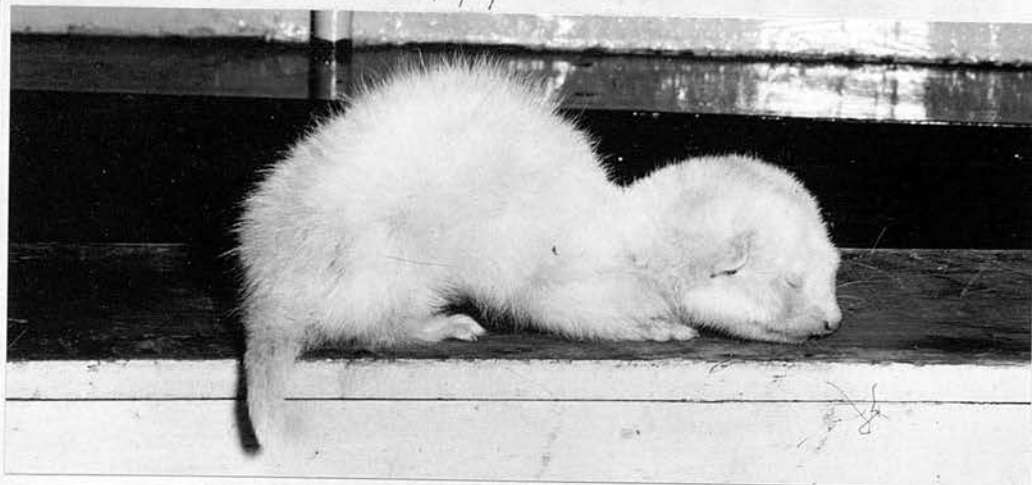
Pair II. Two animals were inoculated intra-peritoneally with 0.5 ml. of tissue culture fluid from the HEP 2 propagated Edmonston strain of the measles virus. These animals were observed daily for the next four weeks and remained afebrile and perfectly normal; they did not differ in any respect from the control animals.

Pair III. Two animals received 0.1 ml. of measles virus intra-cerebrally contained in a 20 per cent ferret brain emulsion. The inoculum in this case was prepared from the young ferret which, in the previous experiment, was sacrificed 18 days after receiving measles virus and cortisone. One of these animals died 24 hours after inoculation; death was assumed to be traumatic and it was discarded. The second animal became gradually lethargic and twelve days later was emaciated with a hunched back and ruffled fur. The head appeared to be disproportionately large and bulging in the region of the parietal bones. There was a purulent discharge from the conjunctivae. There was, however, no pyrexia and the animal fed normally. The animal was observed for a period of ten weeks when it was photographed (A) and compared with the control animal (C). Its weight was 190 gms. compared with 480 gms. the weight of the control. The animal was now sacrificed

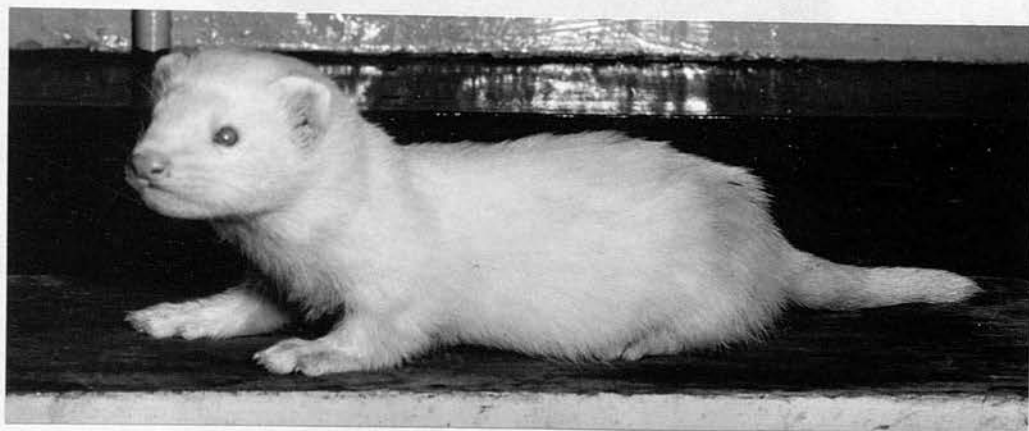


and an autopsy performed. Macroscopically the principal abnormalities were confined to the brain; the heart, lungs, viscera and other tissues showed little abnormality. When the skull was opened, the brain was seen to be congested and the convolutions of the cerebral hemispheres were flattened. On horizontal section the cerebro-spinal fluid under pressure escaped and the lateral ventricles were so greatly enlarged and distended with clear fluid that they were bounded above by only a narrow band of brain matter. The appearances were thus those of a marked hydrocephalus. A careful search was made for any lesion at the site of the needle entry but none could be found. Examination of the mid-brain and cerebellum showed only a generalised hyperaemia. Histological examination of sections of the cerebral cortex showed a generalised hyperaemia but there was little inflammatory reaction and only small numbers of mono-nuclear cells had invaded the tissue. The remaining organs showed no abnormality except that the bladder mucosa appeared thickened.

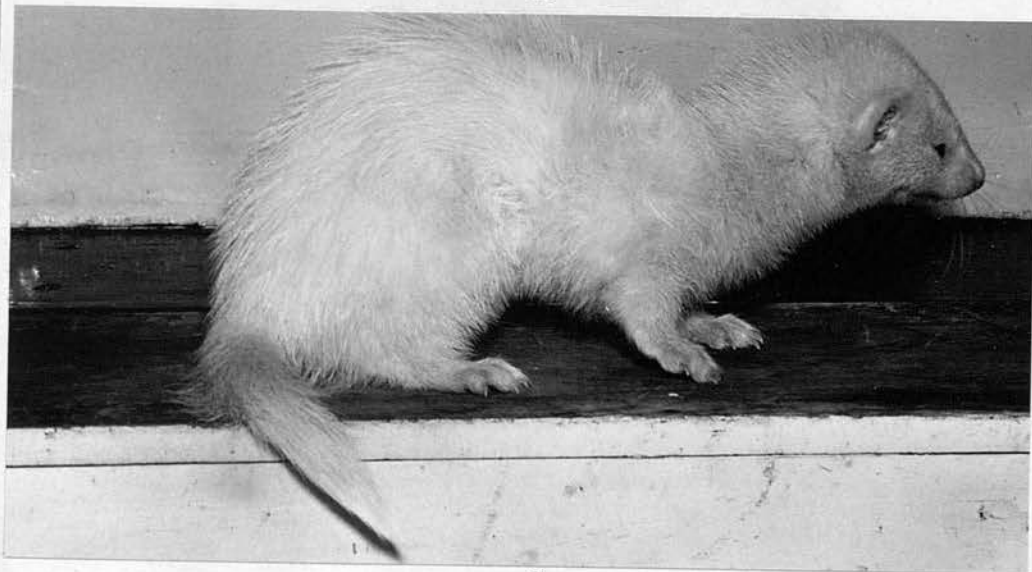
It was difficult to decide whether these effects had been due to the measles virus or the results of trauma; certainly the lesion had been situated at the interventricular



A

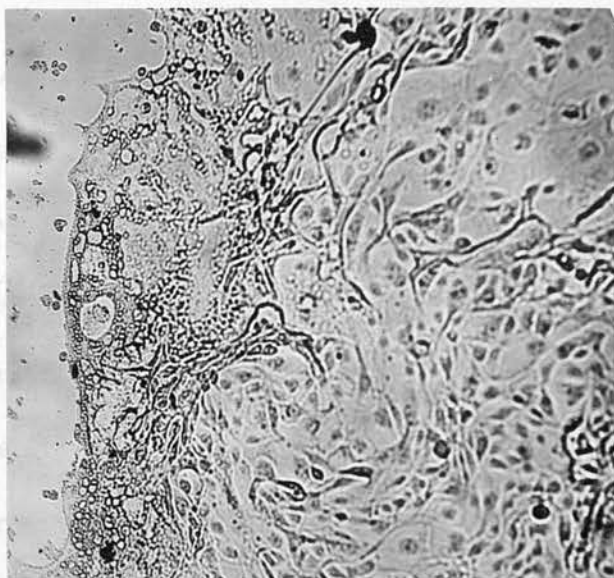


B

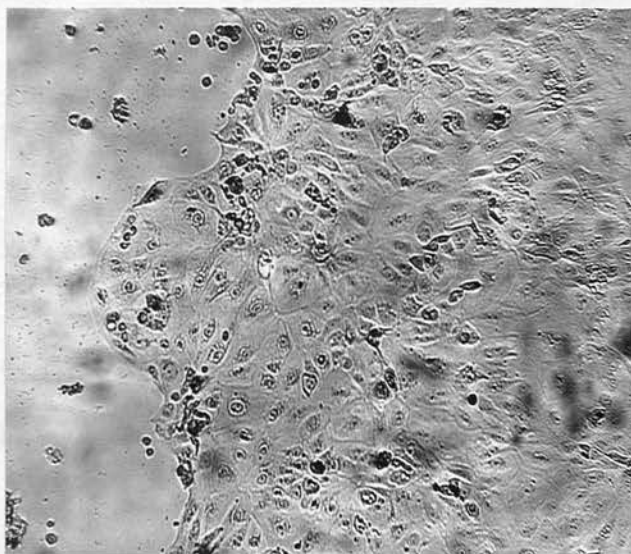


C

Three male ferrets from the same litter. A, 10 weeks after intra-cerebral inoculation with measles virus. B, 10 weeks after intraperitoneal inoculation with the same virus. C, uninoculated control.



Human amnion cells (x80) showing measles cytopathic effect three weeks after inoculation with a suspension of ferret brain.



Normal human amnion cells (x80) which were uninoculated controls in the above experiment.

foramen.

It was unfortunate that no further suckling ferrets were available to carry out more passage experiments.

However, the measles virus was recovered from the brain of this animal in human amnion tissue cultures and its identity was established by the fact that its cytopathic effect and syncytium formation was neutralised by the reference measles anti-serum.

Thus from this experiment it can be concluded that the measles <sup>Virys</sup> does persist in viable form in the brain tissue of suckling ferrets for periods up to ten weeks.

Pair IV. Two animals were inoculated intra-peritoneally with 1 ml. of the 20 per cent brain emulsion which was used for Pair III. Seven days later they were found to be lethargic, dragging their hind limbs a little, and taking much longer to right themselves when turned over on their backs as compared with the control animals. The weight of these animals (photograph B) was 340 gms. as compared with 480 gms., the weight of the control animal (photograph C). One of these animals was bled by cardiac puncture at this time and the blood was found to contain the measles virus when five days after inoculation to tissue cultures of human



amion cells a typical cytopathic effect was observed. As in Pair III the virus was identified in neutralisation tests.

Young male guinea-pigs weighing between 250

550 gms. were used for the production of infection. The

inoculum was prepared as follows:

#### Materials

The incubation, inoculum, and material used

the infection on material and material used

#### Discussion

The first batch of guinea-pigs was infected with

the infection of the guinea-pigs was of the

strain, but after the second batch the

all the guinea-pigs were infected with the

infection of the guinea-pigs was of the

The experiment was repeated, but this time

the guinea-pigs were infected with the

strain. The results were as follows:

Results of the experiment are as follows:

Results of the experiment are as follows:

Results of the experiment are as follows:

Results of the experiment are as follows:

Results of the experiment are as follows:

Results of the experiment are as follows:

Effect of Measles and distemper vaccination in guinea pigs.

Young male guinea-pigs weighing between 450 to 530 gms. were used for the production of antisera for both measles and distemper viruses.

Measles

The inoculum, dosage and schedule have been given in the section on material and methods (page 65).

Distemper

The first batch of guinea-pigs were given a subcutaneous injection of the egg-adapted strain of canine distemper virus, but after the second subcutaneous injection 10 days later all the guinea-pigs were found dead the next morning - and this was attributed to anaphylaxis.

The experiment was repeated, but this time using the canine distemper virus grown in ferret kidney tissue culture. The maintenance medium used was the same as for measles; the titre of the viruses, and the schedule of inoculations given, again approximated that used for measles.

It was observed that the guinea-pigs inoculated with measles virus appeared to be perfectly normal, put on weight, and in all respects were comparable to uninoculated controls.

The distemper inoculated guinea pigs, however,

behaved differently. Three to four days after the second subcutaneous injection, (i. e. corresponding to the incubation period in ferrets), these guinea-pigs appeared to be sick, and were off food. Two died on the 14th and 17th days after the first injection was given. The rest recovered, but never became normal, their coat remained ruffled, the back arched, and the food consumed was much less. There was also a yellow secretion from the eyes.

In the following table the gain or loss of weight of the guinea pigs after the final bleeding (12 weeks) is shown.

Table to show weight, loss or gain, of guinea pigs inoculated with the measles distemper viruses.

	Guinea pigs	Starting Weight (Pre inoc.)	Final weight	Gain or Loss of wt.
Measles	1	462.0	623.5	+ 161.7
	2	417.0	642.0	+ 125.0
	3	523.5 gms.	750.5	+ 127.0
	4	510.5	623.5	+ 113.0
Distemper	5	475	340.2	- 134.8
	6	480.5	358.6	- 121.9 died
	7	530.0	423.6	- 106.4
	8	510	408.6	- 101.4

All weights are given in grams.

(One of the distemper guinea-pigs died after ether anaesthesia).

## Adaptation of the Viruses to suckling mice

### Measles

Virus grown in HEP 2. cells and mice aged from a few hours to 5 days were used.

The inoculated tissue culture tubes, when showing maximal cytopathic effect were quickly frozen at  $-70^{\circ}\text{C}$  and thawed at  $37^{\circ}\text{C}$ , on two occasions. This fluid was harvested, pooled and centrifuged for 5 minutes at 4000 R. P. M. A portion of this supernatant was titrated for infectivity in HEP 2. cells (Titre  $10^4$  TCID<sub>50</sub>/ml.) and the rest was stored at  $-70^{\circ}\text{C}$  and used for inoculation.

Inoculations were made with a tuberculin syringe, into parietal lobes of infant mice, the dosage employed being 0.01 ml. per mouse.

In harvesting the brains of mice, a wide-bore needle specially ground to form a short bevel was attached to a 20 ml. syringe. After the mice were anaesthetised, the needle was introduced into the cranial cavity and brain material aspirated. By manipulating the needle, the entire brain substance could be extracted. This method was found to be easier, quicker and had less chance of bacterial contamination because the entire process was done in a 'closed



operation' manner.

Brain suspensions were made with serum broth; to 1 gm. of Brain, 9 volumes of serum broth was used to make a 10 per cent W/V suspension of infected mouse brain. The following table summarises the experiment.

TCF	18hrs.	9 / 8
TCF	24hrs.	8 / 12
TCF	36hrs.	4 / 10
P 1	12hrs.	4 / 8
P 2	24hrs.	10 / 11
P 3	36hrs.	7 / 8
P 4	24hrs.	6 / 10
P 5	48hrs.	12 (34 + 74)
P 6	36hrs.	9 / 9

TCF - Through

Micro-dialysis with

Table to show the adaptation of measles virus in

suckling mice.

TCF or Passage	Age of Mice	No. affected No. inoculated	Day on which effect was seen	Type of Effect	Remarks
TCF	5dys.	0 / 11	-	-	Observed for 3 wks.
TCF	48hrs.	0 / 8	-	-	Do.
TCF	24hrs.	0 / 12	-	-	Do.
TCF	3hrs.	4 / 10	7 to 9th day	Spastic paraly- sis	3 were canabalised
P 1	12hrs.	4 / 7	7 to 8 dys.	Deaths (3) Spastic paraly- sis (4) Killed	3 deaths in 72hrs. canabalised
P 2	>12hrs.	10 / 12	7to8 days 10mice	2 died..	in 24 hrs.
P 3	3hrs.	5 / 6	6 dys.	Spastic paraly- sis	One canabalised
P 4	24hrs.	6 / 10	5-6 days 4mice	Spastic paraly- sis	2 canabalised
P 4	48hrs.	12(5a + 7b)	a) 7dys. b) 5dys.	a)spas- tic paraly- sis b)Hyper- activity	
P 4	36 - 48 hrs.	9 / 9	6-7 days	Spastic paraly- sis.	

TCF = Tissue culture fluid P<sub>1</sub> = passage on.

Mice dying within 24hrs. of inoculation were regarded as\*traumatic deaths.

### Distemper

Similar procedures were used to infect suckling mice with the wild strain (II) of the distemper virus. A ferret spleen emulsion was used and the identity of the virus it contained was checked by infecting ferrets with it and by neutralisation tests in mice. It proved impractical to estimate the infective titre of the ferret spleen emulsion because only a limited number of ferrets were available.

The Protective Effect of Vaccination with Measles and Distemper Viruses in Ferrets.

Experiments were carried out in an attempt to determine whether previous vaccination with the measles virus conferred any protection on the ferret against challenge with the distemper virus.

The ferrets used were as follows:

Group 1 Two adults ferrets which had received three injections of measles virus (see p 66 ). They had been used for the production of measles antisera and at the time of the experiment their sera contained complement fixing antibodies at titres of 1 in 8 and 1 in 16 but no antibodies to the distemper virus.

Group 2 Three suckling ferrets which had been inoculated intracerebrally with measles virus and which had reacted subsequently (see p. 173. ).

Group 3 A single young adult ferret which had developed a paraplegia and incontinence 7 days after intracerebral inoculation with measles virus (see p. 175).

Group 4 Two young adult ferrets which had previously received 1 ml. subcutaneously of the avirulent egg-adapted EPIVAX vaccine strain of canine distemper virus.



Group 5 Two normal healthy ferrets.

All the animals in each of the four groups were challenged by the subcutaneous inoculation of 1 ml. of the C. D. 9. distemper virus. The freeze dried ampoules of ferret spleen suspension obtained as a reference from Burroughs Wellcome were used for this purpose: the preparation had an L. D. 50. of  $10^{4.2}$ .

The result of these experiments are seen in Table

1	M	1	1 in 2	4 weeks	100%
2	M	2	1 in 4	4 weeks	100%
3	M	3	1 in 16	4 weeks	100%
4	M	4	1 in 64	4 weeks	100%
5	M	5	1 in 256	4 weeks	100%
6	M	6	1 in 1024	4 weeks	100%
7	M	7	1 in 4096	4 weeks	100%
8	M	8	1 in 16384	4 weeks	100%
9	M	9	1 in 65536	4 weeks	100%
10	M	10	1 in 262144	4 weeks	100%
11	M	11	1 in 1048576	4 weeks	100%
12	M	12	1 in 4194304	4 weeks	100%
13	M	13	1 in 16777216	4 weeks	100%
14	M	14	1 in 67108864	4 weeks	100%
15	M	15	1 in 268435328	4 weeks	100%
16	M	16	1 in 1073741312	4 weeks	100%
17	M	17	1 in 4294965248	4 weeks	100%
18	M	18	1 in 17179860992	4 weeks	100%
19	M	19	1 in 68719443968	4 weeks	100%
20	M	20	1 in 274877775360	4 weeks	100%
21	M	21	1 in 1099511101440	4 weeks	100%
22	M	22	1 in 4398044405760	4 weeks	100%
23	M	23	1 in 17592177623040	4 weeks	100%
24	M	24	1 in 70368710492160	4 weeks	100%
25	M	25	1 in 281474841968640	4 weeks	100%
26	M	26	1 in 1125899367874560	4 weeks	100%
27	M	27	1 in 4503597471498240	4 weeks	100%
28	M	28	1 in 18014389885992960	4 weeks	100%
29	M	29	1 in 72057559543971840	4 weeks	100%
30	M	30	1 in 288230238175887360	4 weeks	100%
31	M	31	1 in 1152920952703549440	4 weeks	100%
32	M	32	1 in 4611683810814197760	4 weeks	100%
33	M	33	1 in 18446735243256791040	4 weeks	100%
34	M	34	1 in 73786940973027164160	4 weeks	100%
35	M	35	1 in 295147763892108656640	4 weeks	100%
36	M	36	1 in 1180591055568434626560	4 weeks	100%
37	M	37	1 in 4722364222273738506240	4 weeks	100%
38	M	38	1 in 18889456889094954024960	4 weeks	100%
39	M	39	1 in 75557827556379816099840	4 weeks	100%
40	M	40	1 in 302231310225519264399360	4 weeks	100%
41	M	41	1 in 1208925240902077057597440	4 weeks	100%
42	M	42	1 in 4835700963608308230389760	4 weeks	100%
43	M	43	1 in 19342803854433232921559040	4 weeks	100%
44	M	44	1 in 77371215417732931686236160	4 weeks	100%
45	M	45	1 in 309484861670931726744944640	4 weeks	100%
46	M	46	1 in 1237939446683726906979778560	4 weeks	100%
47	M	47	1 in 4951757786734907627919114240	4 weeks	100%
48	M	48	1 in 19807031146939630511676456960	4 weeks	100%
49	M	49	1 in 79228124587758522046705827840	4 weeks	100%
50	M	50	1 in 316912508351034088186823311360	4 weeks	100%
51	M	51	1 in 1267650033404136352747293245440	4 weeks	100%
52	M	52	1 in 5070600133616545411089172981760	4 weeks	100%
53	M	53	1 in 20282400534466181644356691927040	4 weeks	100%
54	M	54	1 in 81129602137864726577426767708160	4 weeks	100%
55	M	55	1 in 324518408551458906309707070832640	4 weeks	100%
56	M	56	1 in 1298073634205835625238828283330560	4 weeks	100%
57	M	57	1 in 5192294536823342500955313133322240	4 weeks	100%
58	M	58	1 in 2076917814729337000382125253328960	4 weeks	100%
59	M	59	1 in 8307671258917348001528501013315840	4 weeks	100%
60	M	60	1 in 33230685035669392006114004053263360	4 weeks	100%
61	M	61	1 in 13308274014267756802445601621305280	4 weeks	100%
62	M	62	1 in 53233096057071027209782406485221120	4 weeks	100%
63	M	63	1 in 213092384228284108839129625940884480	4 weeks	100%
64	M	64	1 in 852369536913136435356518503763537920	4 weeks	100%
65	M	65	1 in 3409478147652545741426074015054151680	4 weeks	100%
66	M	66	1 in 13637912590610182965704296060216606720	4 weeks	100%
67	M	67	1 in 54551650362440731862817184240866426880	4 weeks	100%
68	M	68	1 in 218206601449762927451268736963465707520	4 weeks	100%
69	M	69	1 in 872826405799051709805075031853862830080	4 weeks	100%
70	M	70	1 in 3491305623196206839220300127415451320320	4 weeks	100%
71	M	71	1 in 13965222492784827356881200509661805281280	4 weeks	100%
72	M	72	1 in 55860889971139309427524802038647221125120	4 weeks	100%
73	M	73	1 in 223443559884557237710103208154588884500480	4 weeks	100%
74	M	74	1 in 893774239538228950840412832618355538001920	4 weeks	100%
75	M	75	1 in 3575096958152915803361651330473422152007680	4 weeks	100%
76	M	76	1 in 14300387832611663213446605321893688608030720	4 weeks	100%
77	M	77	1 in 57201551330446652853786421287574754432122880	4 weeks	100%
78	M	78	1 in 22880620532178661141514568515029901772849280	4 weeks	100%
79	M	79	1 in 91522482128714644566058274060119607091397760	4 weeks	100%
80	M	80	1 in 366089928514858578264233096240478428365591040	4 weeks	100%
81	M	81	1 in 146435971405943431305693238496191371346236480	4 weeks	100%
82	M	82	1 in 58574388562377372522277295398476548538494720	4 weeks	100%
83	M	83	1 in 234297554249509490089109181593906194153978880	4 weeks	100%
84	M	84	1 in 937190216998037960356436726375624776615915520	4 weeks	100%
85	M	85	1 in 3748760867992151841425746905502499106463662080	4 weeks	100%
86	M	86	1 in 1499504347196860736570298762200999642585464960	4 weeks	100%
87	M	87	1 in 5998017388787442946281195048803998570341859840	4 weeks	100%
88	M	88	1 in 23992069555149771785124780195215994281367439360	4 weeks	100%
89	M	89	1 in 95968278220599087140499120780863977125469758720	4 weeks	100%
90	M	90	1 in 38387311288239634856199648312345590850187903360	4 weeks	100%
91	M	91	1 in 15354924515295853942479859324938236340075161280	4 weeks	100%
92	M	92	1 in 61419698061183415769919437299752945360300645120	4 weeks	100%
93	M	93	1 in 2456787922447336630796777491990117814412025600	4 weeks	100%
94	M	94	1 in 9827151689789346523187110367960471257648102400	4 weeks	100%
95	M	95	1 in 39308606759157386092748441471841885030592409600	4 weeks	100%
96	M	96	1 in 157234427036629544370993765887367540122369638400	4 weeks	100%
97	M	97	1 in 62893770814651817748397506354947016048947814400	4 weeks	100%
98	M	98	1 in 251575083258607270993590025419788064195791257600	4 weeks	100%
99	M	99	1 in 1006300333034429083974360101679152256783165030400	4 weeks	100%
100	M	100	1 in 4025201332137716335897440406716609027132660121600	4 weeks	100%

From these results it is seen that the 24th day of this experiment the virus had reached a stage where virus did not cause a fatal infection.

Table Showing the effect of a challenge dose of distemper virus on ferrets immunised against measles (M) and distemper (D) and normal control animals.

Group	Imm. Against	Ferret No.	Titre of measles C. F. antibody.	Interval between last immunising injection and challenge	Result
1	M	1 2 3	1 in 8 1 in 8 1 in 16	4 weeks	death in 18dys. " " 19" " " 18"
2	M	1 2 3	Not done	10 weeks	death in 10dys. " " 10" " " 12"
3	M	1	1 in 16	10 weeks	death in 14 dys.
4	D	1 2	Nil Nil	10 weeks	Survived Survived
5	C	1 2	Nil Nil		death 10th day death 11th day

From these results it is seen that under the conditions of this experiment previous vaccination with measles virus did not protect ferrets against distemper.

Results of complement fixation tests in animals  
immunised with measles antigen

Animal	No. of injections given	Interval between last injections and bleeding	Reciprocal C. F. titres with antigens of -	
			measles	distemper
G. pigs No. 1.	3	10 days	16	neg.
No. 2.	3		8	neg.
No. 3.	3		8	neg.
No. 4.	3		8	neg.
No. 5.	3		16	neg.
No. 6.	3		32	neg.
Rabbit(1)				
Pre Inoc.	-	-	4	16
Post Inoc.	(3)	10 days	64	16
Booster	(4)	10 days	256	16
Rabbit(2)				
Pre inoc.	-	-	4	8
Post inoc.	-	10 days	64	32
Booster	3	10 days	256	16
Ferrets 1.	3	10 days	8	neg.
2.	3		16	neg.
3.	3		4	neg.
4.	3		8	neg.
5.	3		8	neg.
6.	4	10 days	16	neg.
7.	4		16	neg.
8.	4		16	neg.

In all animals except rabbits the pre. inoculation  
sera were negative for both measles and distemper  
antigens.

Result of complement fixation tests in animals  
immunised with distemper antigen and dogs with  
naturally occurring disease.

Animals	No. of injection given	Interval between test inj. and bleeding	Reciprocal C.F. titres with antigen of -	
			measles	distemper
Dogs	1	Convalescent	8	128
	2	blood	8	64
	3	-	4	32
	4	-	4	32
	5	-	8	128
	6	-	8	128
Ferrets *				
	1		4	32
	2	3	2	32
	3	10	2	32
	4		2	64
Rabbit(3)				
pre inoc.		-	-4	8
post inoc.	3	10 days	4	128
			16	256
Rabbit (4)				
pre inoc.			4	16
post inoc.	3	10 days	32	64
Booster (IV)			32	256

Pre-inoculation serum samples of all animals except those rabbits were negative for both. Rabbits were obtained from the animal breeding station, Bush, Edinburgh. The other animals were bred in this laboratory.



From the foregoing two tables it can be seen that after the immunisation of ferrets and guinea pigs with the measles and distemper viruses complement fixing antibodies were produced only to the homologous virus and not at all to the heterologous antigen. However, in dogs convalescent after naturally occurring distemper, the development of high titres of antibodies to the distemper virus was accompanied by the appearance of low levels of antibodies to the measles virus.

The results of complement fixation tests carried out on the rabbits immunised against the two viruses present a somewhat different picture and it should be noted that the serum of these animals taken before inoculation contains low levels of antibodies to both viruses. Again the highest level of antibody was attained against the homologous antibody. The four rabbits used in this experiment were obtained from the small animal breeding station outside Edinburgh and had not as far as could be determined been exposed to either virus. The presence of antibodies in their serum before the experiment began destroyed the value of the results but the finding may indicate the existence of natural antibodies or virus inhibitors in some stocks of rabbits.

## SECTION V

### Comparative serological studies

In the first four sections of these results a study has been made of the effects of infection with the measles and distemper viruses; natural infections in man and animals were observed and experimental infections of animals with both viruses were reported. During this work antibody estimations were carried out at suitable intervals of time and the results are recorded in detail in each section. For the most part the complement fixation technique was employed but neutralisation tests in both tissue cultures and embryonated eggs were also used to check the findings and to supplement the results.

The following table shows the typical findings in two clinical cases of measles. The figures have been taken from results already recorded on pages 114.

Case	Rise of reciprocal of antibody titre			
	Measles virus		Distemper virus	
	Comp. Fixation	Neutralisation in HEP 2.	Comp. Fixation	Neutralisation in eggs.
M. G.	0 to 512	0 to 256	0 to 32	0 to 16
J. A.	0 to 64	0 to 256	0 to 16	0 to 32

Neutralisation in OVO of the Distemper virus

Sera	Nil	1 : 4	1 : 8	1 : 16	Serum dilutions			1 : 256
					1 : 32	1 : 64	1 : 128	
Nil (control eggs)	3/3							
<u>Measles</u>								
Human J. A. acute	3/3	3/3	NT.	N. T.	NT.	NT.	NT.	NT.
Human J. A. conv.		NT.	0/3	0/3	2/3	3/3	3/3	NT.
Human M. G. acute		3/3	NT.	NT.	NT.	NT.	NT.	NT.
Human MG. conv.		NT.	0/3	0/3	3/3	3/3	3/3	NT.
Rabbit reference (BW)		NT.	0/3	0/3	0/3	2/3	3/3	NT.
<u>Distemper</u>								
Canine Dog I		NT.	0/3	0/3	0/3	1/3	3/3	3/3
Horse reference (BW)		NT.	0/3	0/3	0/3	0/3	0/3	1/3

Numerator shows number of eggs showing lesions on the allantoic membrane.

Denominator indicates number of eggs inoculated.

NT. = Not tested.

Virus dose = 200 EID 50 Canine distemper EPIVAX strain.

Doubling serum dilutions made in medium 199.

Incubation 1 hour at room temperature.

Table to show the haemagglutination titre (HA) of Measles Virus (MV), grown in Human Amniotic Cells (HAM), and HEP-2 cells, compared with Distemper Virus (CDV), grown in Ferret Kidney Cells (FKC) and on chorio-allantoic membrane (CAM).

VIRUS		Reciprocal of Antibody titre
M. V.	H. A. M.	64
	HEP-2	8
C. D. V.	F. K. C.	-
	C. A. M.	-

Table to show the comparative titres of complement fixing antibody (CF) and Haemagglutination responses (HA) of three children and three dogs with naturally acquired measles and distemper respectively.

		Reciprocal of Antibody titres		
ANTISERA		C. F.		H. A.
		Measles	Distemper	
M. G.		512	8	64
J. A.		64	32	16
A. B.		64	-	16
Dog	1			-
	2			-
	3			-



These findings in clinically accepted cases of measles together with observations on many single samples of serum taken at all stages of the disease indicate that complement fixing and neutralising antibodies reach their peak about three weeks after the onset. Often the antibody titre is 8 to 16 times higher for the measles virus than it is for the distemper virus. It is generally true that when a high level of measles antibody is present a low level can be demonstrated for the distemper virus. However, when the measles antibody is low it is often impossible to demonstrate any activity in the serum against the distemper virus.

There was little difference between the findings obtained by complement fixation and those from neutralisation tests. The results obtained by the two methods were always closely parallel and at no time during the work was there any discrepancy between the two methods.

In dogs suffering from natural distemper a mirror image of the measles findings was obtained. Titres of antibodies in serum taken late in the disease were 8 to 16 times higher against the distemper virus than against the measles virus (see page 191 ). When the ferrets were immunised against distemper they were fully protected against challenge by a lethal dose of the virus and developed

complement fixing antibodies to titres of 1 in 32 and 1 in 64: yet these animals had no demonstrable measles antibody in their sera. Conversely when ferrets were immunised with a measles antigen they developed only anti-measles antibody and were killed on challenge with virulent distemper virus (see pages 189. ).

Thus it is apparent in both natural and experimental infections with both the viruses that high levels of antibodies to the homologous antigen are developed and are usually but not invariably accompanied by the appearance of low anti-body titres to the heterologous antigen.

These findings suggested the possibility that at least two antigens might be shared by the two viruses which differed from each other quantitatively in the amounts of the antigens they contained. With such a contingency in mind it was decided to study the antigenic structure of the two viruses in more detail and compare them using the methods of precipitation in agar gel.

Agar-gel diffusion tests were first used in virology by Jensen and Francis (1953), with influenza virus and antisera. Later, Mansi (1957), used the untreated infected tissues removed from animals suffering from certain virus diseases such as rinderpest, canine distemper, canine hepatitis, as the antigen.

#### Agar-gel

Both New Zealand agar (Davis) and special Agar Noble (Difco) were tried in concentrations varying from 0.7 per cent to 1.5 per cent. These agars were dissolved in distilled water (White, Simpson, Scott. 1961), in normal saline, and in isotonic phosphate buffers of varying pH from 6.8 to 7.6 for the macro methods; and for the micro-methods up to pH. 9. (Rockefeller Annual Report, 1959). It was found that the special agar (Noble Difco) was the better as it gave a very clean and clear gel. However, it did not set as hard as the New Zealand agar.

Sodium azide at a strength of 0.02 per cent (Gray 1961) thiomersal (Scott and Brown, 1961), and phenol (Mansi, 1957) were incorporated in the gel; but as sodium azide (0.08 per cent) was easily available, and it was preferred to phenol which had to be used at a greater concentration (0.5 per cent).

To find out the formula that gave good results, trials

with a known antigen and its homologous antisera were set up in agar-gels of varying strengths and pH. A lapinised vaccinia virus, and its corresponding antiserum were used, in both the macro and micromethods. This test was also repeated using the formula that gave the best results with a known infected dog lung-tissue, and the high titre anti-distemper serum. Each of the following three methods was used:

1.     Macromethod     A 1.5 per cent solution of Difco Noble agar in 0.85 per cent saline containing 0.01 per cent sodium azide and was poured to form a layer 3-4 mm. thick in small (5 cm) Patri dishes. After hardening, wells 6 mm. wide and 5 mm. apart were cut with the help of a template and a cork borer. The floor of each well was sealed with a drop of molten agar.
2.     Micromethod     Microscope slides were cleansed by boiling in a weak detergent (Pyronex) solution and by repeated rinsing with tap and distilled water. They were finally rinsed with ethanol and then acetone before storing in wide mouthed jars.

Slides were pre-coated by dipping them in 0.2 per cent agar in distilled water kept at boiling point; they were



set to drain in an almost vertical position. When dry 2.5 - 3.0 ml. or 1.0 per cent agar was poured on to the slides which were placed horizontally on a level surface; they were allowed to harden in a dust-free atmosphere. Wells 4 - 5 mm. in diameter and 3 mm. apart were cut and the slides were stored before use in a humid atmosphere at 4°C.

Tube Methods (a) A method modified from that of Ouchterlony in which an antigen was placed on the surface of 0.3 per cent agar containing 50 per cent neat immune serum in 5 x 75 mm. tubes.

(b) A second method was used which gave improved results and was more convenient where solid pieces of tissue were used as the antigen. A layer of 0.3 per cent agar was interposed between the immune serum in the bottom of the tube and the antigen which was placed on the surface.

#### Antigens

Measles: Virus grown in HEP-2 and HAM (human amniotic membrane) was used. Both tissue culture harvests were concentrated by dialysis and absorption, as described under the preparation of antigens for complement fixation tests (page 95. ).

The HEP-2 grown virus was also obtained as a pellet by differential centrifugation as described under the prepara-

tion of the virus for electron microscopy, (page      ). The pellet thus obtained was treated further as follows. A smooth suspension of the pellet was prepared in distilled water using approximately 1/100th volume of the fluid started with.

About half of this concentrated suspension was disintegrated with an ultra sonic vibrator (MSE. MULLARD - Probe - 2).

A drop of the suspension was mixed with an equal volume of 0.05 per cent cadmium acetate solution (Crowle 1960).

To another portion a drop of complement (guinea-pig serum with no antibodies to both measles and distemper virus) was also used but this was discontinued because it did not aid in the diffusion of specific antibodies. A mouse brain extract was also used; this was prepared by sucrose acetone extraction and was also used as the antigen in complement fixation tests.

### Distemper

Animal tissues: Pieces of lung removed from dogs dying of distemper or killed when in extremis, as well as lymph glands and lungs removed from distemper infected ferrets killed in the moribund stage. These tissues were cut into fine pieces with which the antigen wells were filled, a drop of saline was also added, and these pieces were gently pressed down to ensure that they were in contact with the

walls of the wells. Instead of saline, 0.05 per cent of Cadmiun acetate solution (Crowle 1960) was also tried.

Small pieces of spleen were also tried; however, the spleen was so congested that the haemoglobin diffused and it made the observation of the precipitation line very difficult; hence it was not used.

Mouse-brain extract was also tried but that too did not give any precipitation.

#### Antisera

Measles Convalescent human measles sera as well as high titre anti-measles immune serum<sup>d</sup> prepared in the animals were used

Distemper Serum obtained from convalescent or chronically ill dogs, as well hyperimmune anti-distemper serum produced in the laboratory were used.

All the sera were used neat, as well as concentrated by dialysis and absorption of water.

In addition to these human gamma globulin, fraction II, (National Chemical Co.) and dog globulin precipitated by half saturated ammonium sulphate, and by 2 volumes of saturated sodium chloride were also used.

#### Preliminary tests to select the suitable antigens and antisera

The following experiments were carried out to screen

the homologous antigen and antisera that gave a good diffusion line.

### Measles

To conserve the antigen these reactions were carried out by the micromethod. A central well - 5 mm. in diameter, was cut and six wells 2 mm. in diameter, equidistant from each other, and 3 mm. from the central well, were punched out with the help of a template.

The central well was charged with the high titre rabbit measles antiserum that gave high titre in C.F.T. and the peripheral wells with different antigen preparations. The best line was seen between the central well and the concentrated HAM grown virus.

Using this antigen in the central well, six more sera were tested, and the best line was seen between the antigen and serum of a rabbit (No. 4.) immunised in the laboratory.

### Distemper

By similar experiments, lymph glands removed from a ferret and lungs of a dog, together with the serum from a rabbit (Rabbit No. 2.) gave the best diffusion line.

### Tube method:

A 0.3 per cent agar (Noble Difco) in normal saline was prepared and cooled to about 45°C; the required serum



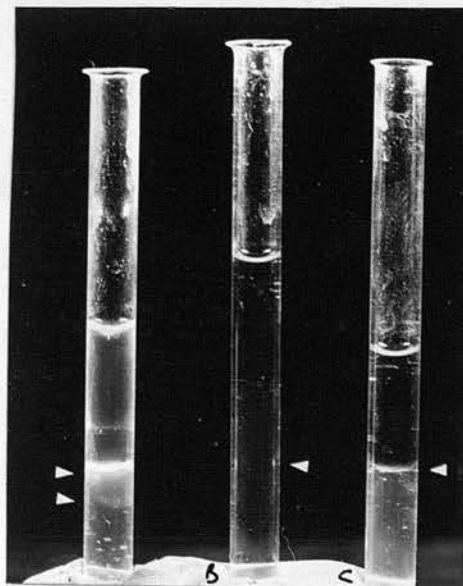
was brought up to 45°C; equal quantities of both were well mixed.

Batch I.

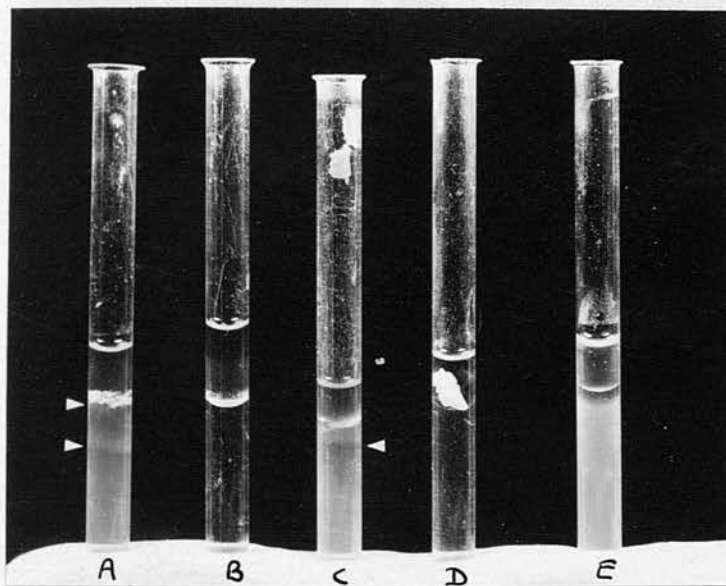
Agar incorporated with high titre rabbit anti-measles serum was poured into tubes to a height of about 1 cm. into three tubes. The tube A was over layered with measles antigen, tube B with control tissue culture fluid, tube C with distemper antigen (pieces of lung covered over with the maintenance medium).

The tubes were stood vertically overnight at room temperature (18° - 22°C). Two diffusion bands were seen in tube A, one broad diffuse band - at the interface and another fainter just below it, but well separated. In the distemper tube C. a thin band was observable. No such bands were evident in the control tube B. Here even the interface was difficult to distinguish. (See photographs below). In this photograph lymph gland piece has been removed to show the interface and the diffusion line.

The arrows indicate the bands.



Batch II. The above experiment was repeated but this time incorporating high titre rabbit distemper antiserum. This was poured into tubes A, C and E. As controls, pre-immunization serum of the same rabbit was used in tubes B and D; tubes B and C distemper antigen and saline; tube C only saline.

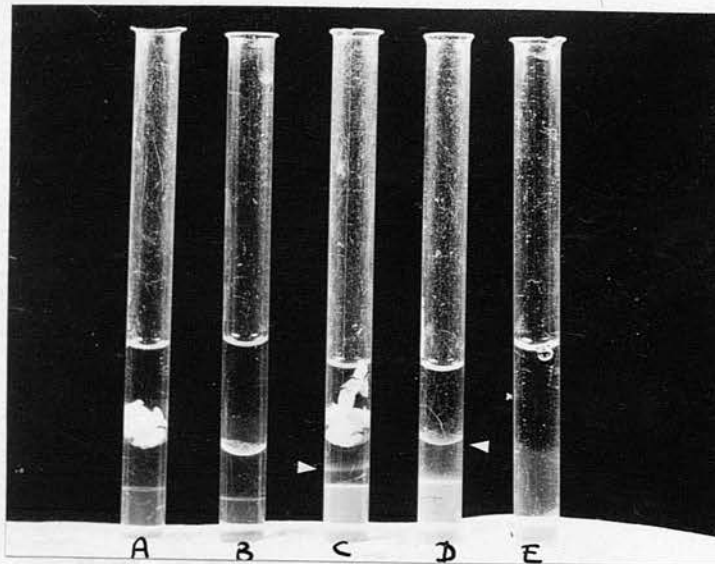


Diffusion line (indicated by arrows) was seen only in tubes containing the antisera and either of the two antigens. The bands appeared in 2 - 4 hours' time. No bands were seen in the control tube.

Batch III. In a third set of tubes a layer of agar was sandwiched between the antiserum below and the antigens above as seen in the photograph on the next page. Tube A and B

had rabbit measles antiserum. Tube E was the control and had only normal rabbit serum.

Tubes A and C had distemper antigen, tubes B and D measles antigen, and E saline in lieu of the antigens.

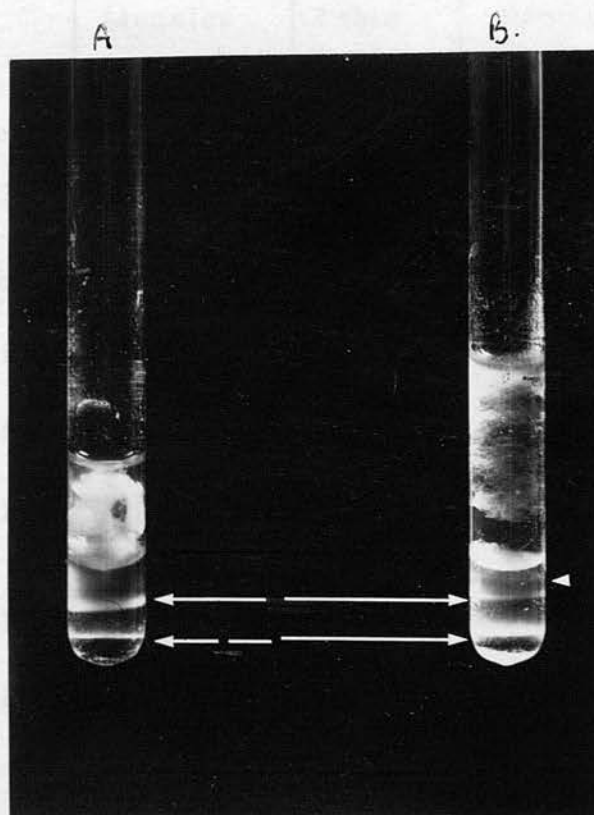


Within 24 hours titres were seen in tubes C and D (distemper antiserum and distemper and measles antigens). In the heterologous combination the band was just at the interface but very soon diffused throughout the agar layer.

In tubes A and B, containing measles antisera, bands developed very slowly and were only faintly evident to the naked eye at 72 hours, when this photograph was taken. It proved impossible to obtain a photographic record of these

at this stage.

These two tubes A and B were left for three weeks in the refrigerator at  $+4^{\circ}\text{C}$  when two definite bands in tube A and three bands in the tube were clearly visible. See photograph below.





## Record of "Bands" seen in immuno-diffusion by the tube method

Observation at the end of

0.3% agar	Antigen	24 hrs.	48 hrs.	72 hrs.
plus				
Anti-measles serum A.	Measles	2 thin bands	diffused	diffused
Anti-measles serum C.	Distemper	1 thick diffuse band	1 diffuse band	diffused
Anti-measles serum B.	Saline	None	None	None
Preinoculation serum B.	Measles	None	None	None
Preinoculation serum A.	Distemper	None	None	None
Anti distemper serum C.	Saline	None	None	None
Anti distemper serum D.	Measles	1 band	1 band	diffuse
Anti distemper serum E.	Distemper	1 thick band	1 band	diffuse
"Sandwich" Method				
Measles anti serum + agar	Measles	2 bands 1 faint	2 bands	2 bands
Measles anti serum + agar	Distemper	1 faint band	1 band	2 bands

cont'd.

Record of 'Bands' seen in immuno-diffusion by the tube methodcontinued.

Observation at the end of

0.3% agar	Antigen	24 hrs.	48 hrs.	72 hrs.
plus				
Measles anti serum + agar	Saline	None	None	None
Control serum + agar	Measles	None	None	None
Control serum + agar	Distemper	None	None	None
Anti distemper serum + agar	Saline	None	None	None
Anti distemper serum + agar	Measles	1 band	1 band	1 band
Anti distemper serum + agar	Distemper	1 band	1 band	1 band

Diffusion lines were seen in the agar layer.

Immuno-Electrophoresis

Shandon Electrophoretic apparatus was used. Veronal buffer at pH. 8.2 was used and the current passed was 50 volts and 15 milli-amperes for two hours.

Agar

At first the agar gel was made with the same

buffer (i. e. at pH 8.2) but the diffusion lines seen were close together, and difficult to distinguish. The agar was then made up with the veronal buffer pH adjusted to 7.2; at this pH and with a concentration of agar of 1.5 per cent, very good separation of the lines was obtained.

#### Method

A clean glass plate 3" square (an old cleaned lantern slide) was coated with 0.2 per cent agar and allowed to dry, as described under micro methods. Two ordinary glass slides were placed on opposing sides to cover about  $\frac{1}{2}$  inch from the margins. The space in between these two slides was filled with the 1.5 per cent molten agar (pH 7.2) and allowed to set.

The two glass slides were then removed and, with a punch, a central trough 3 mm. wide was cut, but the agar strip was left in place (this was made with two safety razor blades and perspex sheet 3 mm. thick). Two wells 5 mm. in diameter were cut on either side of the trough 1 cm. away from the centre of the trough.

The two antigens, measles and distemper, were poured into the two wells, and the current (50 volt + 15 milliamps) was passed for 3 hours. The agar strip was then removed and the trough thus formed was filled with the measles anti-

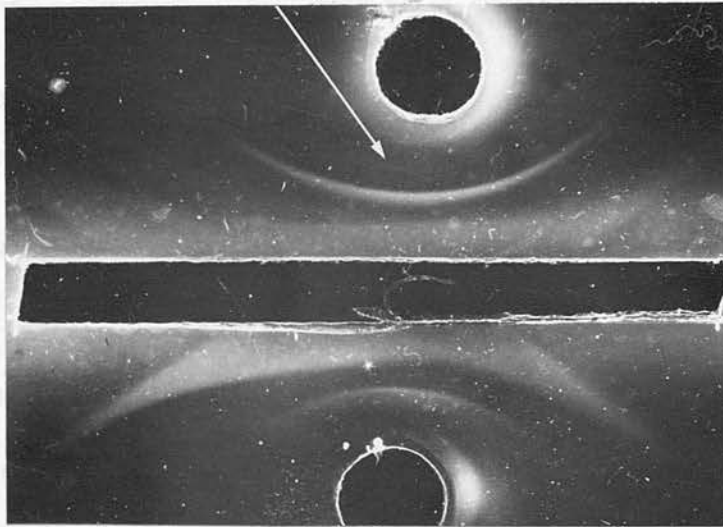
serum in one and the distemper antisera in the other, and left overnight at room temperature.

The slides were soaked in repeated change of buffer saline and left overnight in distilled water in the refrigerator. By this time, except for the precipitation lines, the superfluous proteins were washed off. The slide was dried under filter paper in the incubator, and stained with naphthalene black ((Rowle. 1960).

Slide 1.        Measles antiserum in the central trough.

The next day, two diffusion lines were seen between the distemper antigen and the antiserum and three lines between the measles antigen and antiserum. However, with the distemper antigen the wide band seen at the margin of the trough was better defined than the corresponding one seen with measles antigen. But the 2nd line was comparatively smaller and less well-defined, when compared to the corresponding line of the opposite side; which was very clear cut. The third faint line seen nearest to the measles antigen did not appear beside the distemper agar. The slides were left for another 24 hours in the refrigerator when the lines were seen more prominently (see photograph).



Slide I*Measles antigen**Distemper antigen.*Slide II      Distemper antiserum in the central trough.

Only two lines were seen on both the slides, but the lines were more pronounced on the distemper antigen portion especially the thin second line.

SECTION VIDefinition of Nucleic acid content of the Viruses

The fundamental understanding of the growth processes of the measles virus is hampered by the lack of knowledge of the nature of the nucleic acid which it contains. The large size of the virus particle and the presence of large intranuclear inclusions in infected cells suggested to Cooper (1961) that it was a de-oxyribonucleic acid containing virus but Waterson et al (1961) on the basis of an electronmicroscopical investigation of a rod-like internal component of the measles elementary body were inclined to believe that it is more likely to contain ribonucleic acid similar in structure to that of the larger myxoviruses of mumps and Newcastle disease.

It seemed that further information might be obtained on this problem if virus growth could be arrested by a known inhibitor of the synthesis of nucleic acid. It is recognised that 5-fluoro-deoxy-uridine (F. U. D. R.) effectively inhibits D. N. A. synthesis in animals (Bosch, Harbers, and Heidelberg 1958) and in micro-organisms (Cohen et al 1958) by blocking the conversion of deoxyuridylic acid to thymidylic acid. Furthermore, Salzman (1960) has shown that the growth of the *r*raccinia virus, a known deoxyvirus, is inhibited by F. U. D. R. and that the poliovirus, a proved ribovirus, is quite unaffected.

Thus it appeared possible that an indirect test could be evolved which would yield useful data on the nature of the nucleic acid content of the measles virus.

As a preliminary test a series of HeLa cell monolayers in culture tubes was inoculated in a checker-board pattern with increasing concentrations of F. U. D. R. (50 & 100  $\mu$ gm. per ml.) in one direction with falling dilutions of virus in the other. In addition to the measles virus a known deoxy-virus and one known riboviruses were included in the experiment. The results (Table 218) show that F. U. D. R. had marked inhibitory action on the measles and vaccinia virus and that it did not affect the poliovirus type I.

The experiment was repeated using an extended series of F. U. D. R. concentrations (0.1 to 30  $\mu$ gm per ml.) and the measles and poliomyelitis viruses (Table 216, 17). Again the growth of the measles virus was completely inhibited, even by 0.1  $\mu$ gm F. U. D. R. per ml. while the poliovirus was completely unaffected. The inhibitory effect in this experiment was also tested by infectivity experiments when subcultures to fresh host cells were made in addition to observing the cytopathic effect. In some cases F. U. D. R. may inhibit the production of infectivity without diminishing the cytopathic

effect; this, however, did not occur in these experiments and infective virus was detected in the tissue culture fluids taken from the tubes showing a cytopathic effect.

In a third experiment (Table page 217 ) which was repeated twice, similar results were obtained in the completely different host cell system of secondary monkey kidney tissue cultures. Again there was complete inhibition of the measles virus and the type I poliovirus was unaffected. Vaccinia virus was inhibited when the inoculum was small and when the higher concentrates of F. U. D. R. were employed. Thus high inocula of vaccinia virus can overcome F. U. D. R. inhibition probably because growth is rapid enough to make use of preformed intra-cellular pools of thymidylate.



# CYTOPATHIC EFFECT OF VIRUSES ON HeLa CELLS

Concentration of 5 fluoro-deoxy-uridine in  $\mu\text{gm}$  per ml tissue culture fluid

		Nil.	0.1	0.3	1.0	3.0	10	30
Tube No.		1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3
Log. dilutions of virus <u>Measles</u>	1.0	3 2 2	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
	2.0	1 2 3	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
	3.0	2 2 1	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
	3.5	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
	4.0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
Poliovirus <u>Type I.</u>	3.0	3 3 3	3 3 3	3 3 3	3 3 3	3 3 3	3 3 3	3 3 3
	4.0	3 3 3	3 3 3	3 3 3	3 3 3	3 3 2	3 3 3	3 3 3
	5.0	3 3 3	3 3 3	3 3 3	3 3 3	3 3 3	3 3 3	3 3 3
	5.5	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
Uninoculated <u>cell controls</u>		0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0

Table to show the inhibitory effect of an extended series of fluorouridine concentrations on the growth of measles and poliomyelitis viruses

3 = Marked cytopathic effect; 2 = Medium effect; 1 = Minimal definite effect.

## CYTOPATHIC EFFECT OF VIRUSES ON MONKEY KIDNEY CELLS

Concentration of 5 fluoro-doexy-uridine in  $\mu\text{gm}$  per ml tissue culture fluid

		Nil.	0.1	0.3	1.0	3.0	10	30	50
Tube No.		1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3
Log. dilutions of virus <u>Measles</u>	1.0	3 2 2	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
	2.0	2 2 2	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
	2.5	1 1 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
	3.0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
<u>Poliovirus</u> <u>Type I</u>	3.0	3 3 3	3 3 3	3 3 3	3 3 3	3 3 3	3 3 3	3 3 3	3 3 3
	4.0	3 3 3	3 3 3	3 3 3	3 3 3	3 3 3	3 3 3	3 3 2	3 3 3
	5.0	3 3 3	3 3 3	3 3 3	3 3 3	3 3 3	3 3 3	3 3 3	3 3 3
	5.5	2 3 3	2 3 2	3 2 2	2 3 3	3 3 3	3 3 3	3 2 3	3 3 3
<u>Vaccinia</u>	2.0	3 3 3	3 3 3	3 3 3	3 3 3	3 3 2	2 1 0	1 0 0	0 0 0
	3.0	3 3 3	3 3 3	3 3 3	3 3 3	1 3 1	0 1 0	0 0 0	0 0 0
	4.0	3 3 3	3 3 3	3 2 1	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
	5.0	1 0 1	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0
<u>Uninoculated</u> <u>cell controls</u>		0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0	0 0 0

Table to show the inhibitory effect of fluoro-uridine on the growth of three viruses in monkey kidney cells.

3 = Marked cytopathic effect; 2 = Medium effect; 1 = Minimal definite effect.

## ACRIDINE ORANGE STAINING TO STUDY THE CYTOPATHIC

## OF MEASLES

Cytopathic effect of viruses  
on Hép. 2 cells.

In order to obtain further information as to the nature of  
the nucleic acid in the virus, the infected cells were stained  
with acridine orange and examined microscopically.

		0	50ugm	100ugm
Tube number		1 2 3	1 2 3	1 2 3
Log dilution (x10) of virus <u>Measles</u>	1.0	3 3 3	0 0 0	0 0 0
	1.5	3 3 3	0 0 0	0 0 0
	2.0	3 3 3	0 0 0	0 0 0
	2.5	3 3 3	0 0 0	0 0 0
	3.0	2 2 2	0 0 0	0 0 0
	3.5	0 1 0	0 0 0	0 0 0
<u>Vaccinia</u>	1.0	3 3 3	3 0 0	2 0 0
	2.0	3 3 3	0 0 0	0 0 0
	3.0	3 2 2	0 0 0	0 0 0
	4.0	0 1 2	0 0 0	0 0 0
	5.0	0 0 0	0 0 0	0 0 0
	6.0	0 0 0	0 0 0	0 0 0
<u>Poliovirus Type I</u>	2.0	3 3 3	3 3 3	3 3 3
	3.0	3 3 3	3 3 3	3 3 3
	4.0	3 3 3	3 3 3	3 3 3
	5.0	3 3 3	3 3 3	3 3 3
	5.5	0 0 0	0 0 0	0 0 0
<u>Uninoculated cell controls</u>		0 0 0	0 0 0	0 0 0

Table to show the inhibitory effect of fluoro-uridine

on the growth of <sup>three</sup> ~~four~~ viruses in Hép. 2 cells.

Key. 3 = Marked cytopathic effect. 2 = Medium effect.  
1 = Minimal definite effect.

ACRIDINE ORANGE STAINING TO STUDY THE CHEMICAL  
OF MEASLES AND DISTEMPER

In order to obtain further evidence as to the nature of the nucleic acid in the two viruses, coverslip preparations of infected tissue cultures with suitable controls were stained with acridine orange and examined microscopically with ultra violet light illuminations.

Uninfected tissue cultures of HEP 2 cells and ferret kidney cells were seen to have a red<sup>d</sup>ish orange content in the cytoplasm indicating the presence of R. N. A. The nucleii were stained green indicating D. N. A. and they contained orange nucleoli. Cells infected with polio virus type I (a known ribovirus) had a red colour in both cytoplasm and nucleus. Cells infected with the vaccinia virus, a known deoxyvirus had green intracytoplasmic inclusion bodies.

In the series of photographs shown in the following pages, the cover-slip preparations were examined at 18 hours and 36 hours after infection; to study the spread of measles antigen in the cells.

The first photograph shows three normal uninfected HEP cells. The tint of the red and orange colours was much more brilliant when seen under the microscope and a good deal of the red colour has been lost in reproduction.



The following photographs show giant cell formation and green inclusion bodies which are interpreted as containing D. N. A.

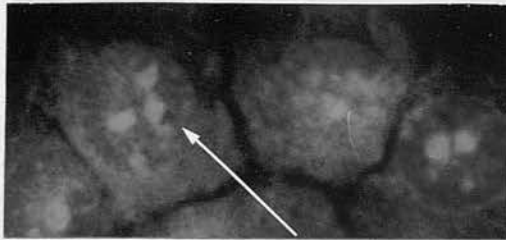
Serial photographs to show the development of the measles virus in HEP-2 cells

All cells stained with acridine orange, and viewed with ultraviolet light. x 800 in all the photographs.



Uninfected HEP-2 cells. Control cells.

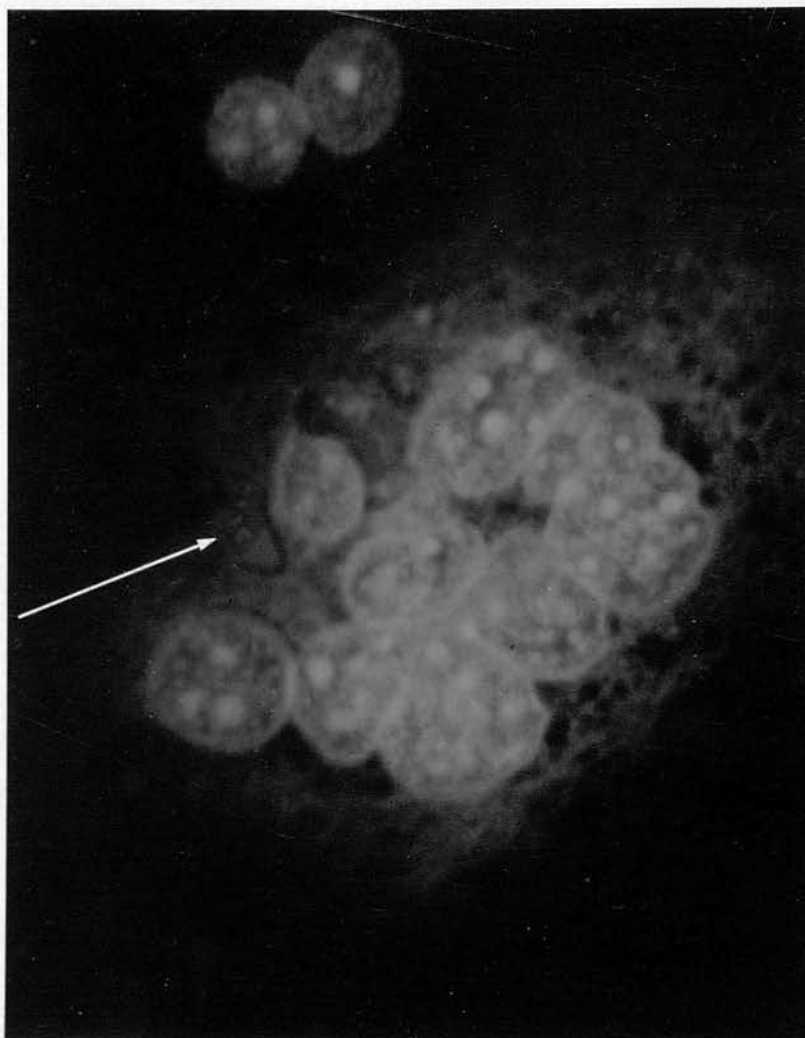
The cytoplasm is stained orange to yellow, with very little green, indicating the presence of R. N. A. In the nucleus a yellow fluorescing thin margin can be made out as well as the yellow fluorescing nucleoli.



16 to 18 hours after infection. Note the intense green fluorescence of the nucleus. The yellow margin is lost and nucleoli have lost their yellow hue. Deep-green fluorescing intranuclear bodies adjoining the nucleoli can be made out. The cytoplasm too is losing its yellow fluorescence.



In these photographs the cytoplasm can be made out faintly and there is overall intensely green fluorescence of both nucleus and cytoplasm.



Hep 2 cells x 800 Preparation, staining and illumination as before. 4 days after infection. Shows a small multinucleate giant cell. The cytoplasm shows the generalised green fluorescence and two large apple green intracytoplasmic inclusions.

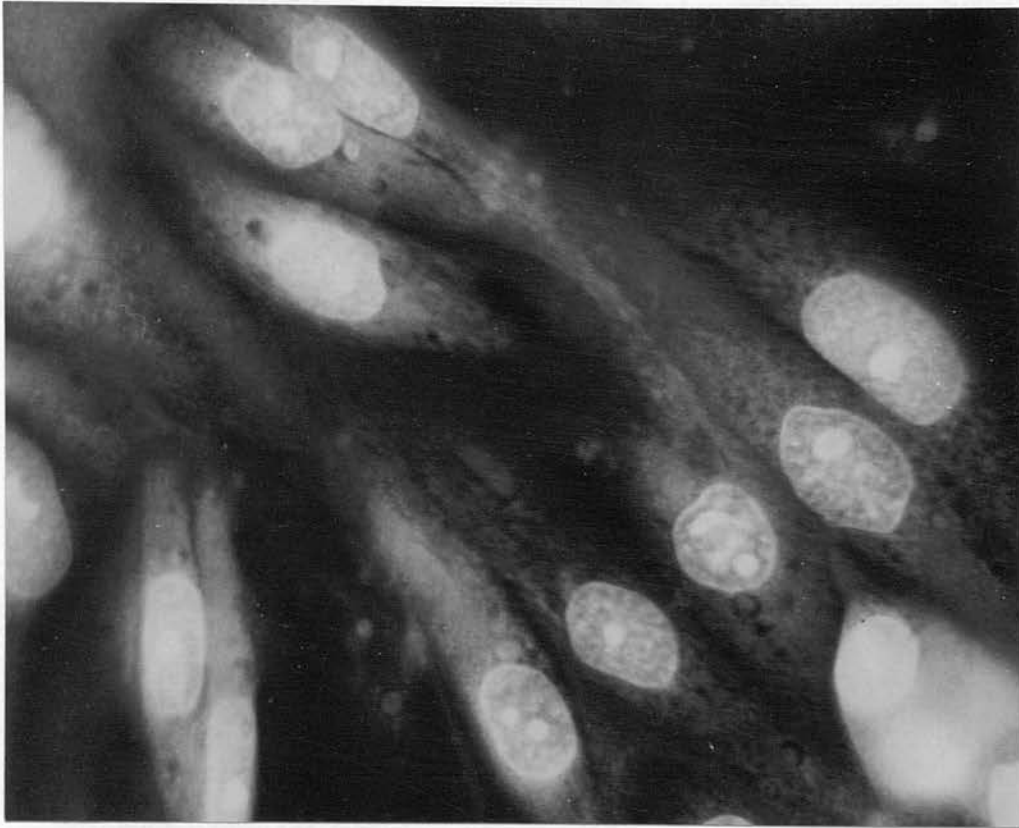


Hep 2 cells x 800 Preparation and illumination similar to the previous one, showing a large intracytoplasmic inclusion.



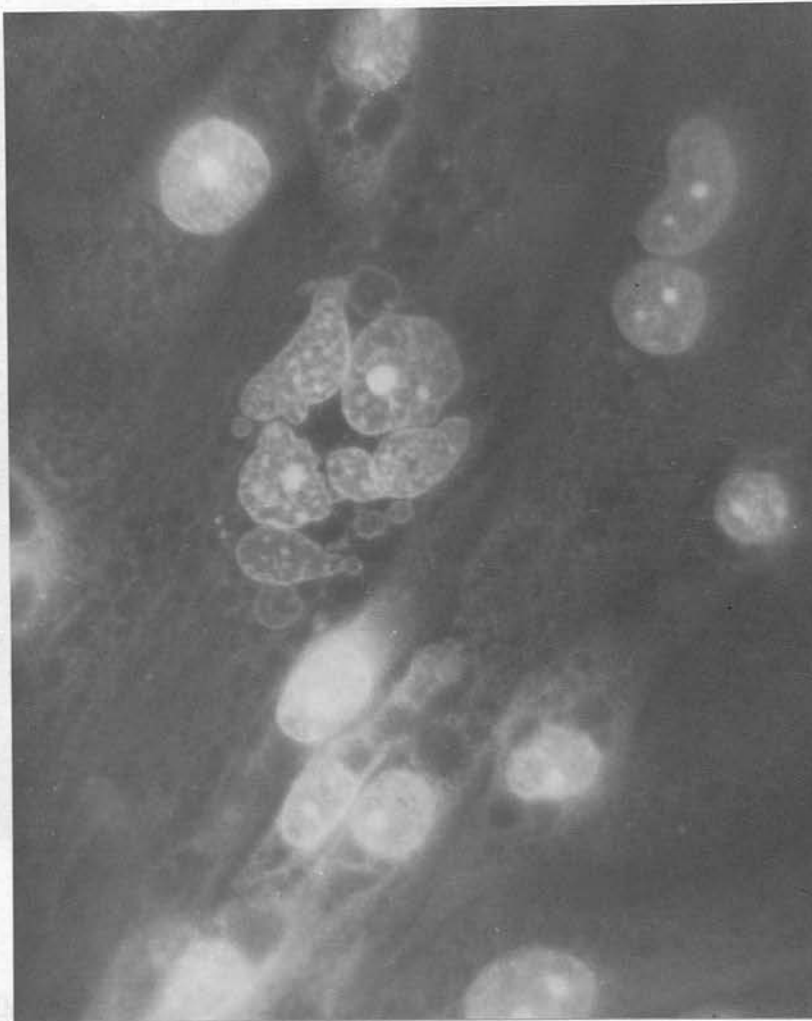
Similar findings were obtained with ferret kidney cells infected with the distemper virus. The first of the following photographs gives an almost true representation of the normal cell with the orange R. N. A cytoplasmic content and the green of the D. N. A. containing nuclei.

In the following two photographs the giant cells formed again contain much green stained D. N. A. in the form of the inclusion bodies.



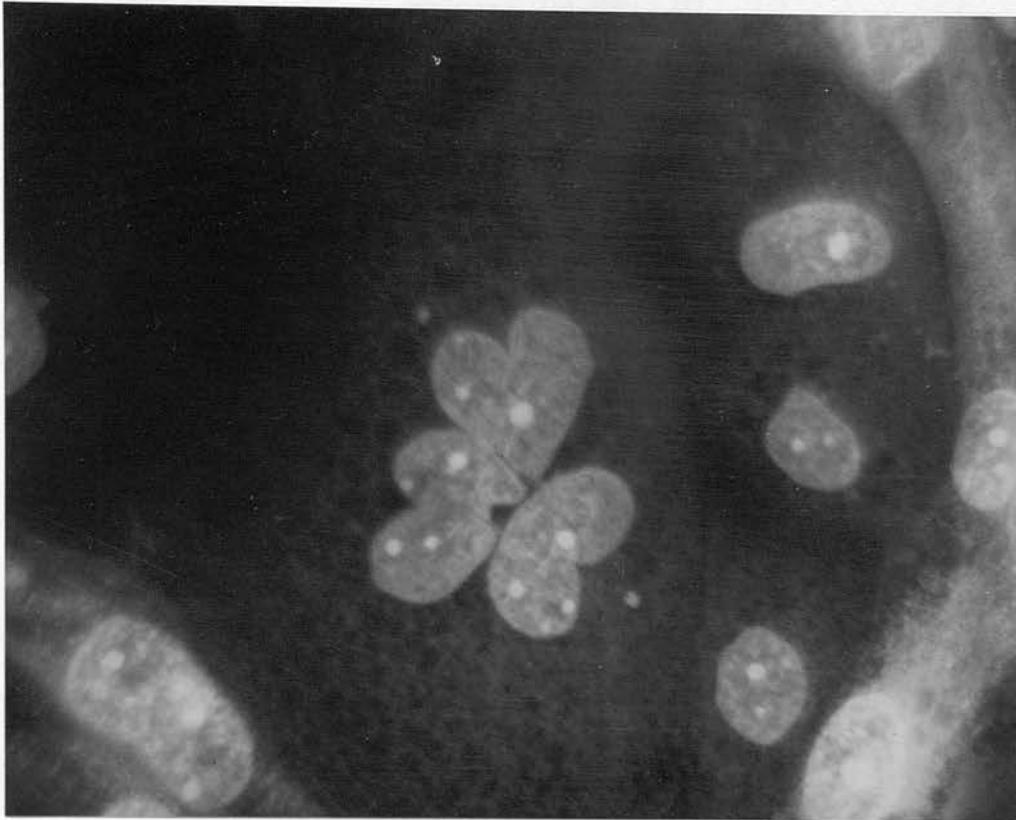
Ferret kidney tissue culture x 800. Control preparation -  
 12 days' growth. Acridine orange staining with ultra  
 violet illumination. Shows the generalised orange to  
 yellow fluorescence of the cytoplasm (R. N. A), yellow-  
 green fluorescence of the nucleii (DNA) the nucleoli  
 fluorescing yellow (RNA).

Infected ferret kidney tissue culture x 800.  
 Cells show the perinuclear fluorescence as before. Note the apple-green cytoplasmic and intranuclear inclusion bodies.



Infected ferret kidney tissue culture x 800

Cells seen near the periphery of the same preparation as before. Note the apple-green intracytoplasmic (C) and intranuclear inclusion bodies (N).

Electron microscopyVirus preparationBresnau and HarcourtInfected cellsand controls

Infected ferret kidney tissue culture x 800. Preparation, staining and illumination identical with the controls, but infected with canine distemper virus 5 days previously. Shows a small giant cell with 6 to 7 nucleii. Note the overall green fluorescence of the cytoplasm and the nucleii, as contrasted with the normal cells. In the three cells on the right side of the giant cell, the perinuclear green fluorescence is well marked.

were made as described previouslycould be seen in most of the cells



SECTION VIIElectron microscopic appearance of the viruses

Virus preparations stained negatively by the methods of Breaner and Horne (1959), Horne et al. (1960), although often satisfactory, are sometimes obscured by the size of the P. T. A. particle grains. Hence P. T. A. dissolved in alcohol was tried. Several concentrations of P. T. A. ranging from 2 per cent to 0.1 per cent, in different strengths of alcohols, absolute 90, 80, 70, 50 and 25 per cent were tried. The best results were obtained with 0.75 or 0.5 per cent of P. T. A. in 50 per cent alcohol in distilled water.

Measles

Measles virus grown in HEP 2 cells was used, as the virus could be grown in bulk in Roux bottles. The HEP 2 cells were easily maintained in the laboratory, unlike the amnion cells, which could not be relied upon as a constant and copious source of host cells.

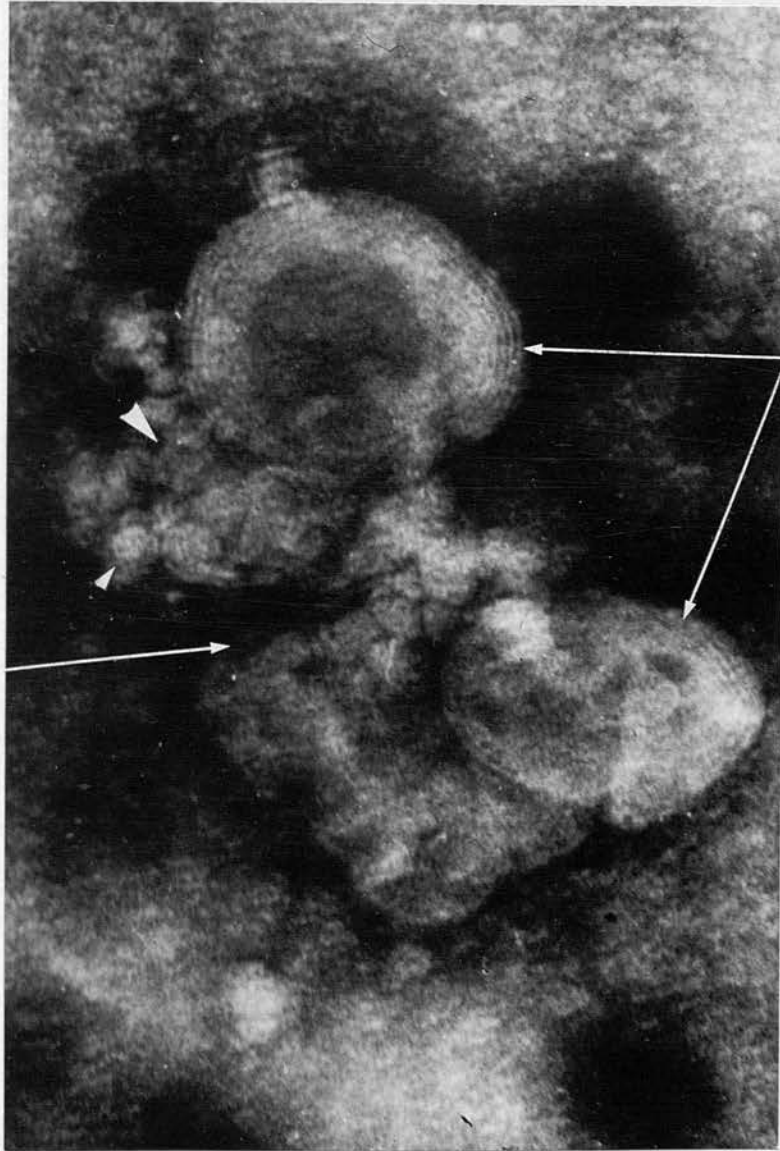
The virus was prepared in several stages of growth. The earliest was when the HEP 2 cells showed the earliest change, i. e. in 48 hours to 72 hours after infection. The cells were detached, broken down, and the virus preparations were made as described (page 87 ). Intact virus particles could be seen in most of the grids viewed.

These virus particles showed an inner central dark mass 90 - 140 mu in diameter, forming a central core. This was surrounded by concentric rings each of which is approximately 5 - 6 mu thick. Two such intact particles ~~X~~can be seen in Electro micrograph No. 1. Adjacent to these are three other particles, distorted and ruptured, probably in a later stage of development; and the irregularly scattered small discoid structures showing a granular surface are also seen. Roughly defining these can be seen what appears to be a membrane outlining and containing these discoid structures.

In the next electro-micrograph No. 2 the "membrane" is seen to enclose a partly disrupted virus particle, still composed of a large number of discoid structures. Below and to the right is seen the "Envelope material". This appears to be the shell of an empty virus particle. The discoid structures are absent and no central core remains. Similar appearances to this were described by Nagington and Horne (1962), for the vaccinia virus and "orf" viruses.

In the third electro-micrograph which was taken at a magnification of 50,000, and the print further enlarged to give a final magnification of 300,000, two herring-bone like

structures can be seen. These correspond closely to the structures which were later described by Waterson et al (1961) in measles, distemper and rinderpest, as well as in other myxoviruses, i. e., Sendai, influenza and mumps.

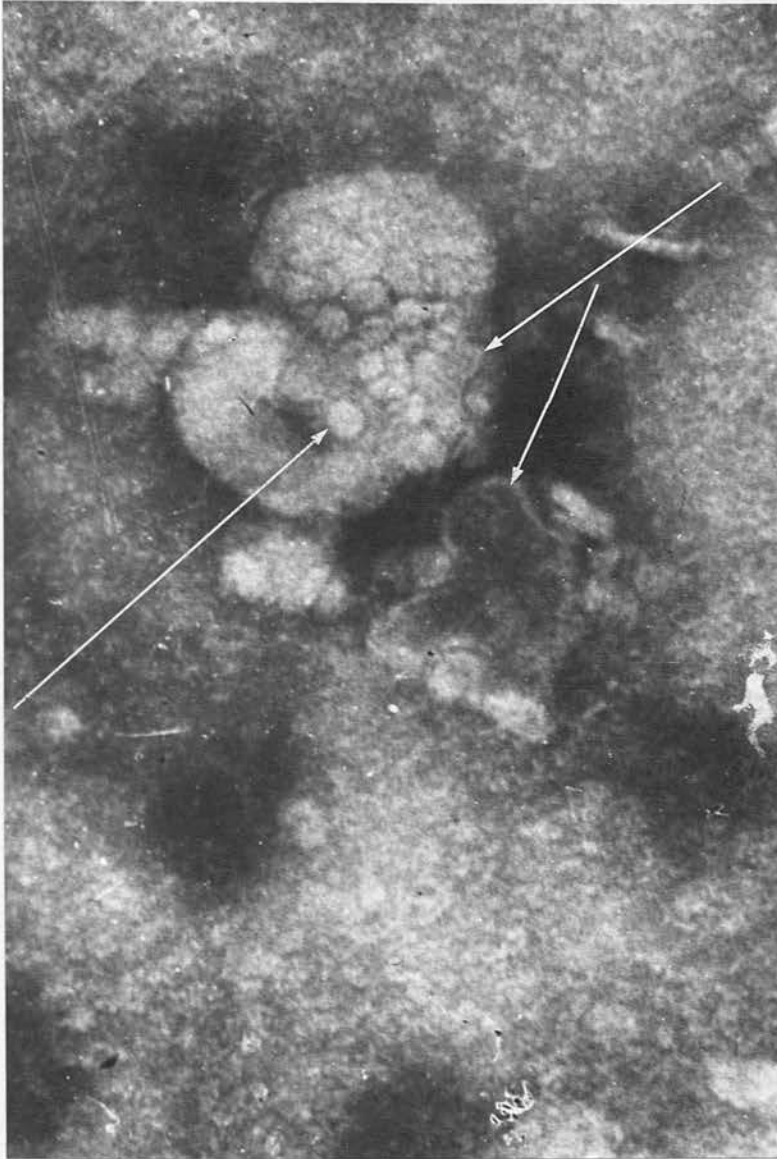


Measles Virus x 150,000

Grown in Hsp-2 cells. 48 hours growth. Phosphotungstic acid negative staining with 0.5 per cent alcoholic solution. The individual Virus particle shows the concentric arrangement. Also seen is a partially ruptured Virus particle, showing the discoid structures

Electron micrograph 3

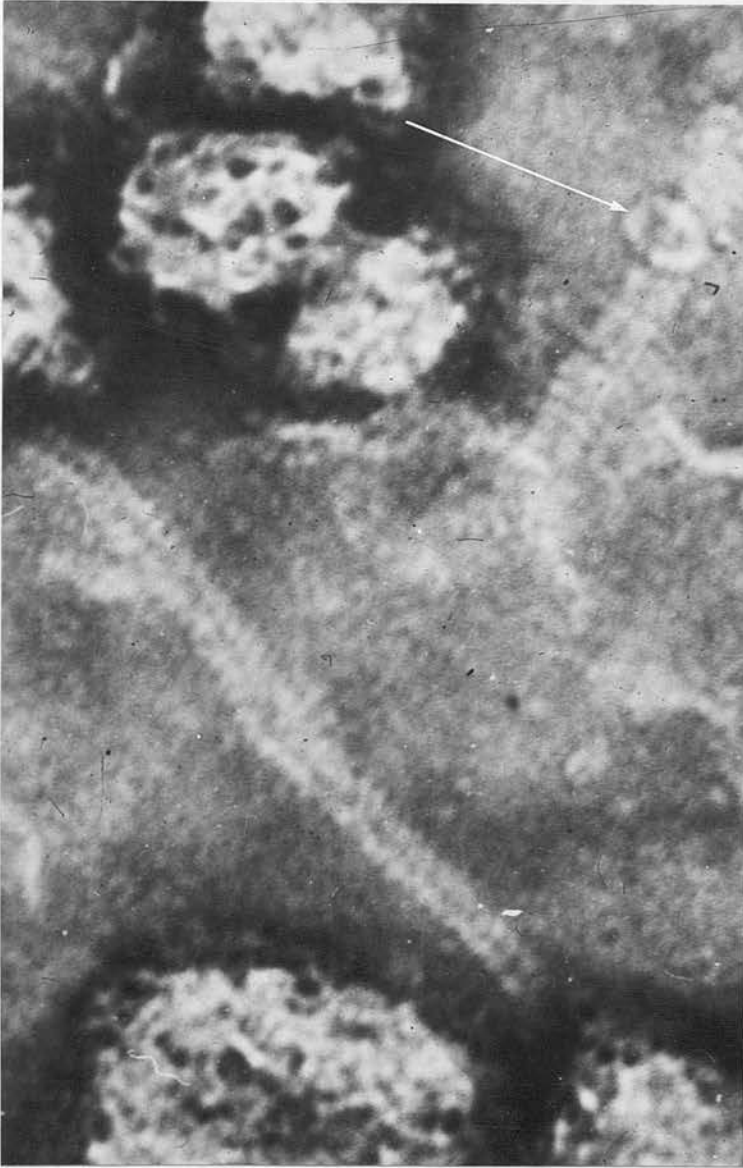




Measles Virus x1000000

Grown in Hep-2 cells. 96 hours growth. One intact Virus particle and another partially disintegrating are seen. Lying beside these is seen, what could be "envelope material". Negative staining with 0.5 per cent alcoholic solution of phosphotungstic acid.

Electron micrograph 1



Measles Virus x300.000

Grown in Hép-2 cells. 96 hours growth. A high magnification photograph to show the "Helix" from a ruptured virus particle. The end on view of one of these helices can be seen as well.

Electron micrograph 2

Distemper

Canine distemper virus grown on the chorioallantoic membrane was prepared for electron microscopy as described and



Measles virus grown in human amniotic cells. 5th day of growth. P. T. A. negative with 0.5% alcoholic solution x 200,000. Note the loosely lying "envelope material", the thickened outer coat and the darkened central mass. The virus particle lying below and to the right, the particle has partly slipped out of the envelope material.

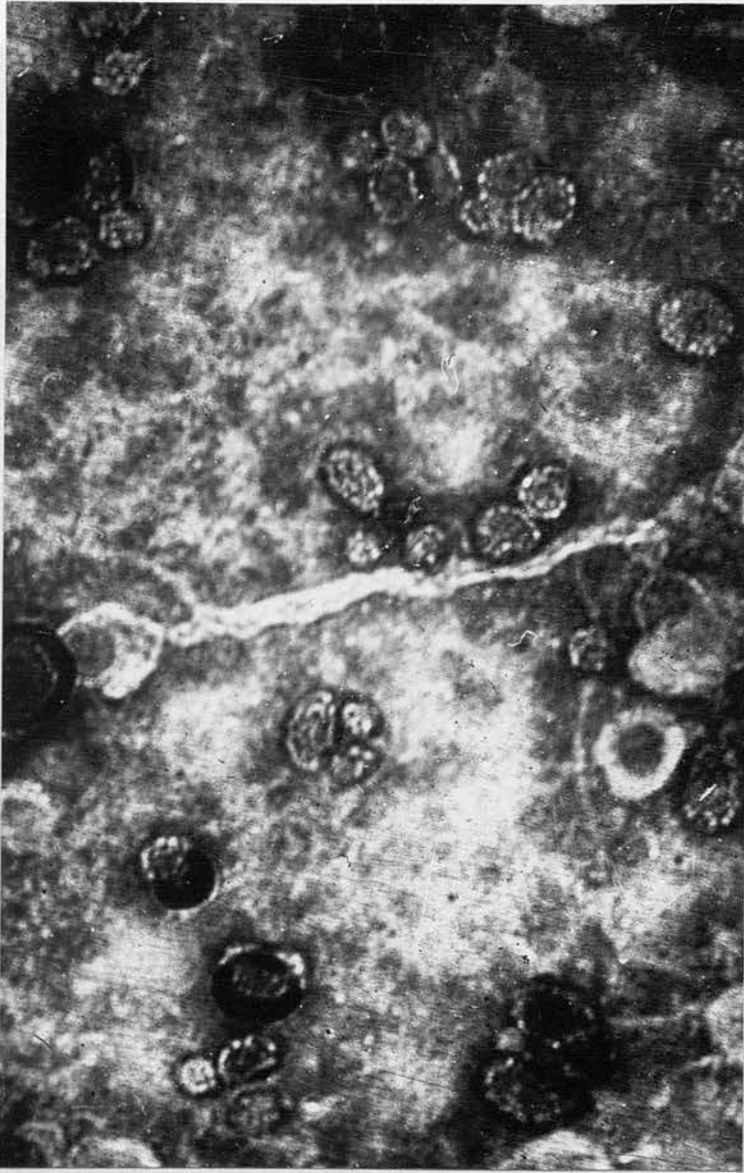
Distemper

Canine distemper virus grown on the chorioallantoic membrane was prepared for electron microscopy as described under methods. Here too, the chorioallantoic membrane was harvested at a very early stage. That is only four days after inoculation. The electro-micrograph (No. 4) presents a picture of such a preparation. The small protuberances seen might be the discoid structures. The next electro-micrograph No. 5 is that of a preparations made from the inoculated chorio-allantoic membrane showing good lesions, and at the usual interval (6 or 7 days) when passages were made for maintenance of strain or preparation of vaccine. This picture shows two partially disrupted virus particles (large arrows) demonstrating the discoid structures, almost identical in appearance to that seen with measles virus. From the lower particle a "helix" is seen extruding from the inside of a mass of discoid structures.

Infected splenic tissue is a good source of virus and is highly infective (Dunkin and Laidlaw, 1946). As far as can be judged from the available literature no attempts have been made to visualise the virus in this situation. An infected spleen was therefore removed, washed and the virus recovered



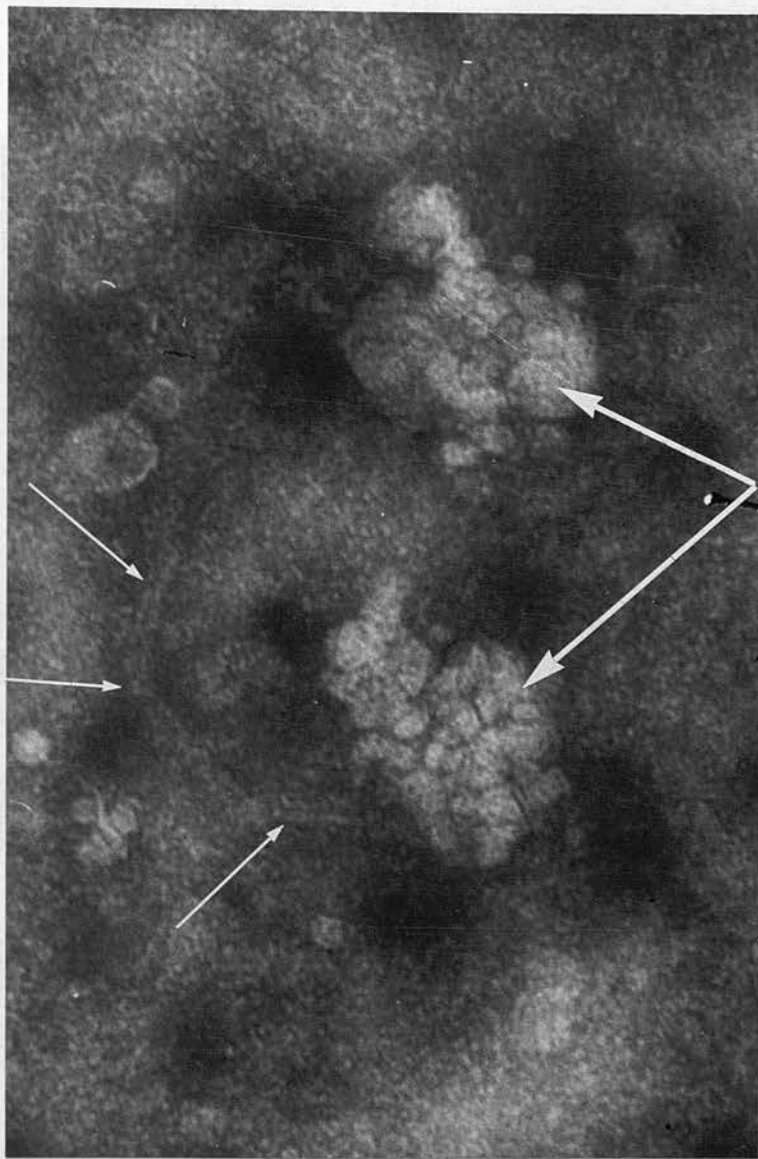
as described (page 90 ). Probably due to the extended cycles of centrifuging and washing, the empty shells of the virus have remained in most of the particle, but in a few a dark core can be made out.



Canine distemper Virus x30.000

Preparation from infected chorio allantoic membrane.  
96 hours growth. Negative staining with phosphotungstic  
acid 2 per cent at pH 7.

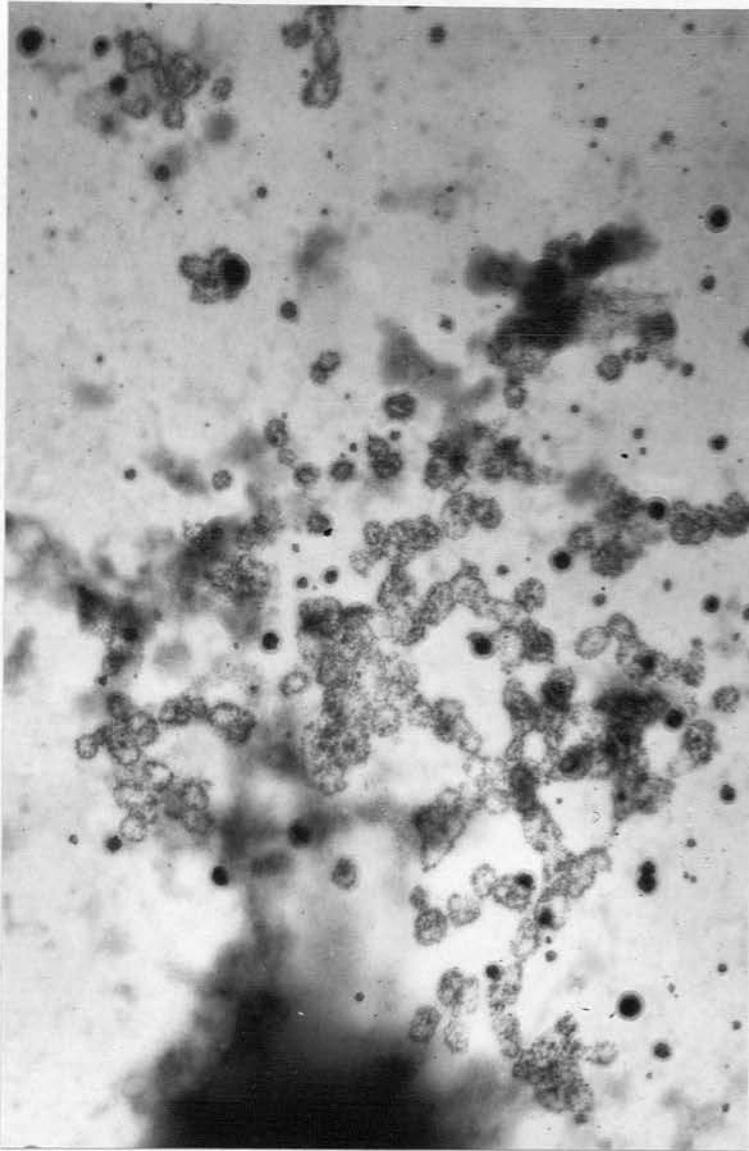
Electron micrograph 6



Canine distemper Virus x80.000

Preparation made from 6 day old lesions on the chorio allantoic membrane. Negative staining with phosphotungstic acid 2 per cent at pH 7, two disrupting particles are seen as well as "helix" from one of them.

Electron micrograph 5

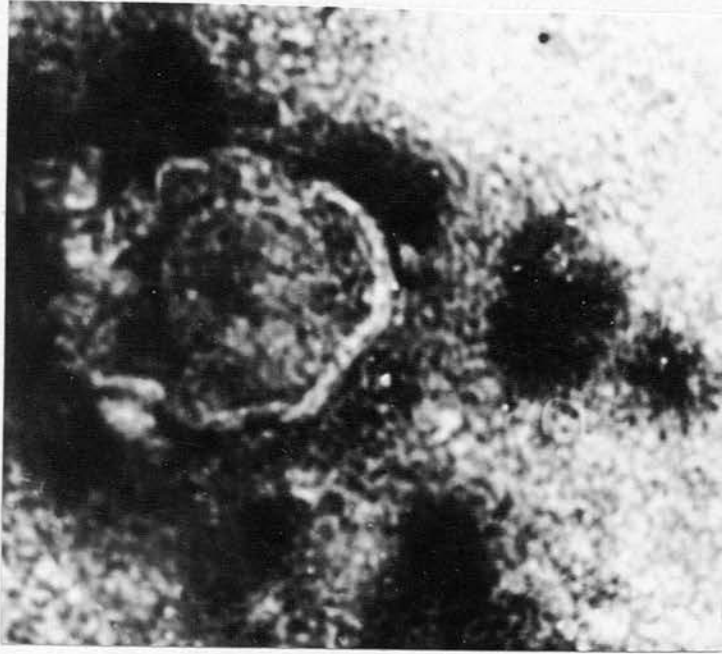


Canine distemper Virus x20,000

Virus recovered from an infected ferret spleen.  
Phosphotungstic acid negative staining with 0.5 per cent alcoholic solution.

Electron micrograph 4





Canine distemper virus - grown in ferret kidney cells.  
5th day of growth. 2% P. T. A. negative staining. The outer  
membrane and the virus particle can be distinctly made out.

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The effects produced by infection with the two viruses.

It was Bryan (1928) who first drew attention to a possible relationship between an upper respiratory disease in humans and the canine distemper. Since then several other workers have attempted to explore the possible connection between giant cell pneumonia, measles and canine distemper. The avenues explored included those of cytology, serology, histopathology, animal and experimental work. These have already been referred to.

Measles is a disease peculiar to man, and has been known from time immemorial. Thus, the virus has a long association with man and no other animal is susceptible to this infection except the monkey. Distemper, however, appears to be of more recent origin (page 9 ). It is not confined to one species, but equally affects several genera of animals, like wolves, (Canidae) foxes (Vulpidae), minks, ferrets, (Mustelidae) racoons, (Procyonidae) to mention a few.

Measles virus is very well adapted to its only host - an almost perfect parasite. In the host it causes a disease of set clinical course and of limited duration, and usually

mild. Complications due to the virus per se are rare. The only important but rare complication is encephalitis. Distemper behaves in an entirely different way. It produces a varied picture, has a prolonged chronic course with a high mortality rate, and in the surviving animals, permanent damage and sequelae often result. So varied is the picture that for a long time it was discussed whether all types of distemper were caused by the same virus or not. Because of extreme polytropism, distemper manifests itself in such varied forms, seldom seen with other viral or other infections. Larin was able to demonstrate that at least three strains of antigenically different types of distemper existed (page 15 ): and he held the view that "there is a progressive and continuous change occurring in the antigenic structure of the distemper virus which cannot be regarded as a stable entity".

Broadly the histopathology of measles can be divided into two phases. The first or pre-rash phase occurs in the early stages of the illness and merges into the second or post-rash phase, roughly at the time when antibodies are beginning to appear in the blood, and when the virus itself disappears from blood and naso-pharyngeal secretions.

In the pre-rash stage the virus is widely distributed and causes two types of giant cells, one type appearing



exclusively in the epithelium and lungs. The other type occurs in all the lymphoid tissues and are the "Warthin-Finkeldey" bodies already referred to (page 161 ). The epithelial giant cells often become detached and appear in the naso-pharyngeal secretions, thus providing, according to Tompkin and Macaulay (1955) a diagnostic feature. This stage apparently is prolonged and exaggerated in cases of giant cell pneumonia in children, in experimentally infected monkeys, and is very marked in those that die before the appearance of the rash.

In the lymphoid tissue, the giant cells, though smaller in size, with a lesser number of nuclei and with absent or only scanty inclusions, are seen several days before the appearance of the rash. They are diffusely distributed throughout the lymphoid tissues, viz. tonsils, thymus, lymph nodes, appendix, Peyer's patches and the spleen. Simon and Ballon (1948), demonstrated these Warthin-Finkeldey cells in tonsils, 5 days before the rash.

Roberts and Bain (1958) came to the conclusion that the giant cells appear about seven days before the rash becomes manifest and that they disappear very shortly after this.

With the appearance of the rash and the coincident

appearance of the antibodies, these giant cells disappear from the tissues, and thus in the post-rash phase a more or less normal histological picture is presented. Thus it is understandable that no giant cells were found in the fatal case of measles described in this report. However, Bolande (1961) has demonstrated the giant cells in the bladder epithelium of a case of measles, that died 7 days after the appearance of the rash.

In cases of measles with no evident clinical encephalitic signs, the involvement of central nervous system has been demonstrated by indirect methods; Robbins (1962) quotes the work of Ojala when the electro-encephalographic tracing showed abnormalities in about 50 per cent of cases, and by the appearance of "leucocytes" in the cerebro-spinal fluid in many cases. The maximal pleocytosis occurred from 5th to 10th day after the rash and may vary from 3 cells to 758 cells per c.m.m. Black, Reissing and Melnick, (1959) in their review, mention the isolation of four strains of measles virus from C.S.F. by Frankel. In the case of measles encephalitis studied in this work, there was definite histopathological evidence of disseminated encephalomyelitis but attempts to isolate measles virus from brain material failed. (The only record of isolation of measles from brain

material is by Shaffer. Rake & Hodes in(1942).

The lesions seen with distemper are more varied but they more or less correspond with pre-rash phase in measles though they are exaggerated. The reticulo-endothelial system and the epithelial tissues are more extensively and seriously involved, and thus there are more complications, and many different clinical manifestations of the disease. The virus has a definite affinity for certain epithelial layers lining the respiratory digestive, vascular and the uro-genital systems. The complication and sequelae depend upon which system is affected the most. In distemper encephalitis the inclusion bodies are not apparent in the nerve cells (MacIntyre et al(1948), but are mainly found in the endothelial cells of meninges, the ependymal cells, the neuroglial cells and the histiocytes.

### The viruses

Measles virus has been isolated and propagated in cells of human and simian origin. Recently dog-kidney cells have been used (Frankel 1957) both for isolation and propagation. In this work it has been shown that the ferret kidney cells also supports the growth of the virus. Distemper virus too can be easily isolated and grown in dog and ferret kidneys. But, all attempts to propagate distemper virus in cells of human and simian origin failed (page 141). Neither is there any record in literature of this being achieved.

The optimum temperature for maximum cytopathic effect and virus yield for measles virus appeared to be  $35^{\circ}$  to  $37^{\circ}\text{C}$  as the virus is inactivated at higher temperatures. Distemper virus, however, in infected ferret kidney cultures incubated at  $39^{\circ}\text{C}$  was shown in this work to give earlier and more marked cytopathic effects (Photographs on page 148-50). These effects may well be a reflection of the natural temperature of the host species. Normal temperatures are for man ( $98.4^{\circ}\text{F}$ ) for dogs ( $101.6$  to  $102.8^{\circ}\text{F}$ ) and for ferrets ( $101.6^{\circ}\text{F}$ ).

The two cell systems common to both the virus in



which they can easily propagate appear to be those derived from dog kidney and ferret kidney. Of these the ferret kidneys were used. The cytopathic changes as observed with the light microscope showed a marked difference. With the distemper virus, a more obvious cytopathic effect was seen at a higher temperature ( $39^{\circ}\text{C}$ ) and much earlier. This consisted of large syncytial masses with up to 150 nuclei. These nuclei were arranged peripherally with or without an additional central cluster of nuclei (photograph on page 148). With the measles virus two types of cytopathic effect were apparent. The giant cells could be described as true syncytia, in that many nuclei were uniformly scattered over the entire area of cytoplasm, and no aggregation of stranding and stellate cells, or the spindle cells. This effect was attributed to the lack of glutamine in the maintenance medium (Reissig, Black, and Melnick (1956)). But the recent work of Seligman and Rapp (1959), attribute it to the characteristics of the host tissue cells, and to the virus itself by Mitus (1962).

Yet another difference in the growth habits of these two viruses is seen in the formation of plaques in chick embryo tissue cultures. The measles virus gives larger plaques (1 - 2 mm. in diameter) and takes a longer time (7 to 12 days) to produce them. Whereas the distemper virus

produces minute plaques (0.5 to 1.0 mm. in diameter in a shorter period of time (2 - 3 days).

There was no great difference detected in cytological studies of growing cells infected with the two viruses. Preparations made at timed intervals during the growth of the viruses showed firstly a diffuse reaction within the nucleus, most easily detected in cells stained with fluorescent dye acridine orange; but also seen with cells stained with phloxine-tartrazine. The process continued until a diffuse green fluorescence appeared in peri-nuclear region and spread gradually throughout the cytoplasm. This was followed by the formation of early giant cells from the fusion of two or three adjacent cells, and at this time definite green fluorescing cytoplasmic inclusion bodies could be seen; these were clearly defined. As time progressed and larger syncytial masses were formed, the inclusion bodies grew in size to a diameter of as much as 2 to 3  $\mu$  and assumed irregular shapes. The inclusion of distemper virus appeared smaller, sharper, generally more clear cut (see photograph on page 149.) and more deeply acidophilic in preparations stained by haematoxylin and eosin or by phloxine-tartrazine. It can also be stated that the sequence of cytopathological events following the distemper virus infection proceeded

more rapidly than with measles virus and that the total effect was much more severe as was witnessed by the greater vacuolation and disorganisation of the cytoplasm.

Throughout the many observations made on infected cells in this work, it was surprising that only on a few occasions was it possible to find the large Cowdry Type A inclusion bodies which Enders and other workers have described. In all the staining techniques employed care was taken to avoid strongly acid fixatives such as Bouins and Zenkers fixatives. The only acid fixative used was Carnoy's fluid for the fluorescent staining and it was only in these preparations that large intra-nuclear masses were seen. From experience with other types of virus inclusions (e.g. molluscum contagiosum) it was felt that acid fixatives produce precipitations and shrinkage effects as well as altering the pH values of the nuclear material. Thus may it well be that the large intra-nuclear masses described by other workers are related to their use of Bovins fixative. The general process of virus growth spreads slowly and diffusely from nucleus to cytoplasm and finally to inclusion body.

The interpretation of electron-micrographs

According to Waterson and his co-workers the measles and canine distemper virus have a helical structure closely resembling that of myovirus. Their virus preparations were made with viruses that were grown in HEP-2 or chick embryonic tissue cultures, until there was a maximum cytopathic effect in the cells. The viruses were stored and transported presumably at  $-70^{\circ}\text{C}$ . However, in the work here described both measles and distemper viruses were examined at very early stages of growth, i. e., at the first sign of cytopathic effect. The measles virus was grown not only in HEP-2 cells but also in human amniotic cells, because it has been shown that the virus grown in these cell layers showed a variation in their complement fixing and haemagglutinating properties. (The distemper virus was grown in eggs as well as in ferret kidney cells and was obtained from infected ferret spleens as well).

This series of electromicrographs show appreciably different appearances to those described by Waterson et al. In the measles virus at a very early stage of growth, the series of concentric rings are very evident, and are more numerous than seen in their photographs. The discoid or



"rosette" structures, a very prominent feature in the present series of electron-micrographs, are seen inside the "envelope material". They appear to lie on the surface of the central core. They can also be seen to a lesser extent in the photographs of Waterson et al who interpret these "rosettes" as "probably segments of the outermost coat rolled up". The limiting membrane or envelope material was well seen in all micrographs.

Thus, in electron micrographs of the measles virus it is possible to distinguish the following components. Externally there is a limiting membrane which in this investigation was of varying thicknesses. In virus grown in human amnion cells it is 9 to 10 m $\mu$  thick and in HEP-2 propagated cells it is appreciably thinner, measuring 5 to 6 m $\mu$ . The latter figures are in agreement with Waterson's findings.

Within the membrane are the striking, concentric rings which are seen in the intact particle and adjacent to the limiting membrane. In disrupted particles it is possible to make out the displaced membrane, which appears as a broken shell from which the internal components are often seen to extrude. In other circumstances the shell is empty and completely void of any content. The internal components cast

loose from the virus particles after the rupture of the membrane consist of helical structure with a periodicity of 5 - 6 m $\mu$  - a finding which agrees closely with that of Waterson et al. In this work, however, a large number of discoid structures, 20 - 40 m $\mu$  in diameter, are seen clearly lying beside the membranes of the ruptured particles. These contrasted with Waterson et al pictures where they are seen only in small numbers. However, these authors used older and more mature viruses which had been kept stored for varying periods.

There are thus four components that can be visualised in the measles virus particles. (It must be added that the distemper virus is morphologically identical). These components are: (1) an outer membrane, (2) the disc like structures, (3) the concentric rings and (4) Helix forming the core.

The interpretation of the nature of each of these components and the manner in which they are assembled to form the complete measles or distemper virus particle, is a matter for conjecture. The authors' own interpretation of his and Waterson's findings lead him to the following hypothesis as being best to fit the facts so far known.

The outer membrane is thicker in amnion cell grown virus, because the virus has been able to stay and grow for a longer period inside the host-cell. Hence it is more mature. The thickness of this envelope material runs parallel with the amount of complement fixing and haemagglutinating titres of the measles virus grown in different cell systems. There is a suggestion in one of the electron micrographs presented here that there are small protuberances or "spikes" resembling somewhat those emerging from the surface of the influenza virus particle in the model and in the electron micrographs of Horne and Wildy (1962). It is also noteworthy that the distemper virus which has no haemagglutinating activity has an appreciably thinner limiting membrane.

The innermost centre of the core of the virus particle is vaguely defined in all the electron micrographs and merges gradually into the formation of concentric rings which are less sharply delineated internally but become very clear cut at the periphery where they are adjacent to the membrane. This appearance could be accounted for if the helix were coiled from a central point and wound in circles of increasing diameter until the limiting membrane is reached. Probably the helix is composed of a central spiral structure of D. N. A. and to its surface are attached the discoid structures which

give the herringbone appearance. The discoid structures may be composed of protein and are possibly analagous to the capsomeres of the tobacco mosaic and other viruses.

Without further observations on the detail of the fine structure of this group of viruses it is not possible to elaborate the hypothesis further. A careful study of ultra thin sections of cells at timed intervals after infection with these viruses has never been undertaken and would be essential if an understanding of the processes of the synthesis and assembly of the virus particle is to be understood. Such a study combined with observation on the appearance of haemagglutinins and complement fixing antigens would be invaluable expecially if combined with observations on the effects of ether disintegration.



Is measles a deoxyvirus or a rebovirus?

The nature of the nucleic acid in the measles virus and distemper is not known. Waterson et al (1961) on the one hand, tend to the view that it may be R. N. A. because their electron-micrographical studies have shown that the viruses contain an internally placed coiled helical structure almost identical in its screw symmetry and pitch with that contained by the known R. N. A. containing myxoviruses. Cooper (1961), on the other hand has placed the measles virus tentatively in his group of "Deoxyviruses".

One important reason for attempting to define the nucleic acid is to acquire information for use in determining the taxonomic position of the measles virus. Earlier systems of the classification of animal viruses were based on habitat and tissue tropisms and predelections at the level of the intact animal, on the types of lesions produced and on the type of vector concerned in transmission. Much difficulty was experienced, and was due partly to the genetic mutability of the viruses themselves and partly due to the environmental and genetic factors which influenced the hosts.

It is natural therefore to seek more stable criteria which are not liable to variation or mutation. One of the

most useful primary criteria in this respect is the nucleic acid content of the virus particle. It is generally accepted that a true virus particle must contain either D.N.A. or R.N.A. (The psittacosis agent which is thought to contain both is for this and other reasons no longer regarded as a "virus" but is classified with the rickettsiae). Furthermore, the possibility that D.N.A. can mutate to R.N.A. or vice versa is extremely remote and can for practical purposes be disregarded.

The series of experiments described above in which the growth of the measles virus was inhibited by 5-fluoro-deoxy-uridine (F. U. D. R.) are sufficiently clear cut to indicate strongly that the measles virus is a D.N.A. containing virus. Other known D.N.A. containing viruses e.g., the vaccinia virus were inhibited and known R.N.A. containing viruses, e.g., the polio-virus, were unaffected. The results were reproducible in two tissue culture systems, HeLa cells and monkey kidney cells.

Although this method of determining the nucleic acid present is indirect, it is one which can readily be applied in the laboratory. Direct methods such as estimating the D.N.A. in concentrated purified virus particles would require very large scale apparatus to produce adequate vol-

umes of the virus for biochemical analysis and even to obtain purified infective nucleic acid from the measles virus susceptible to the specific action of nucleases is scarcely within the scope of any but large laboratories.

A more direct method and one which also permits the study of the development of virus in the cell is that of fluorescent microscopy which was resorted to. Armstrong and Niven (1957) have shown that when virus infected tissue culture cells were stained with acridine orange fluorochrome and were viewed by ultra-violet light, the R. N. A. and D. N. A. containing components fluoresce in different colour. The R. N. A. containing viruses fluoresce shades of yellow to red, and the D. N. A. containing viruses shades of green to yellow. However, the false positives do occur, but these are confined to muco-polysaccharides (Armstrong 1956). Pollard and Star (1962) quote the work of Osterhout with Herpes virus in human amnion cells in which he deduced that the virus contained D. N. A.

Corroborative evidence that the measles virus does contain D. N. A. is obtained from examination of the colour photographs of the measles infected cells stained by the fluorescent dye acridine orange (page 220-221). In these

preparations the normal uninfected cells show red staining R. N. A. diffusely situated throughout the cytoplasm with green or greenish yellow D. N. A. containing nucleii. The nucleoli stain red and contain R. N. A. In measles infected cells the red staining R. N. A. material is lost within a few hours and a diffuse apple green colouration replaces it appearing first in the nucleus and peri-nuclear zone and then diffusing through the cytoplasm. Later when the infection has spread to contiguous cells green-stained intra-cytoplasmic inclusions of varying sizes and shapes are seen within the syncytia that have been formed.

The consistent green staining of the nuclei and cytoplasm of measles infected cells and the continuing absence of any red staining R. N. A. material throughout the entire cycle of infection in any of the large number of preparations of monkey kidney and HEP cells examined indicates that virus and virus inclusion materials contained D. N. A. Control preparations with the vaccinia virus showed only green fluorescence and HeLa cells infected with the polio virus retained the red fluorescence throughout.

Thus two pieces of evidence have been obtained, the inhibition of growth by F. U. D. R., and the green fluorescence with acridine orange, both of which point to the D. N. A. con-



tent of the measles virus.

There seems to be <sup>a</sup> little conflict in these observations with the work of Waterson who relies for his suggestion that the measles may contain R. N. A. (and thus be classified as a myxovirus) on the electron microscopical appearances of the fine structure of the internal helix of the virus particle. Presumably, like the tobacco mosaic virus, the helix contained in the measles and in the myxoviruses, is comprised of a helical core of nucleic acid to which are attached protein subunits and perhaps other structures, too. Since D. N. A. is well recognised as having a doubly wound helical structure there seems no valid objection to such a structure appearing in a D. N. A. virus as well as the known R. N. A. containing viruses. Both R. N. A. and D. N. A. have a helical structure and one might expect that both ribo-viruses and deoxy-viruses could contain helices at their central cores.

## Haemagglutination and Haemagglutination-inhibition

The haemagglutinating property of the measles virus has already been referred to in the introduction (page 45). In this work attempts were made to demonstrate such a property for distemper viruses grown in ferret kidney cell and the egg adapted strains. In both cases, no haemagglutination could be observed with Rhesus monkey red blood cells.

Even with measles, this property varied considerably. It depended upon the tissue cells on which it was grown. The primary amniotic cells gave the highest titre in my hands HEP-2 grown virus much less, and none were seen with the chick embryo adapted strain. As already mentioned, Rosanoff (1961) has pointed to this variation in agglutination grown from different animal sources. The haemagglutination inhibition by human sera and immune sera produced in animals was variable but corresponded to the complement fixing titre though at a lower level - a result which somewhat is at variance with that of Rosen (1961). This discrepancy may be explained by the fact that my results are expressed in terms of the initial dilution of serum used e.g. (EFC. - 1 in 16 or 1 in 32) whereas Rosen's results, in common with current American practice, are probably expressed in terms of the final serum dilution after the

addition of virus and cells. Rosen's figures are almost invariably 4 times higher than mine and a factor of this sort would give a simple explanation of the quantitative differences.

Haemagglutination inhibition was not present in dog distemper convalescent sera and in the hyper immune distemper serum produced in the laboratory animals. Thus it may be said that the haemagglutinating property is inherent and peculiar to the measles virus and the quantity is determined perhaps by the host-cells.

Precipitation lines in agar-gel

Mansi (1957) first demonstrated the precipitation lines in canine distemper virus, using untreated infected tissue as the antigen source and convalescent dog sera.

Stone (1960) used the same method for rinderpest, but his antigen was the clarified centrifuged suspension of lymph-glands. White, Simpson and Scott (1961) demonstrated a common precipitation line between the rinderpest antigen and convalescent distemper sera and vice versa.

Using the method described (page 203 ) and distemper infected dog lungs as the antigen source, a precipitation line was seen between it and a measles anti-serum (convalescent human). A similar line was also obtained with distemper antisera (blood of dog with chronic distemper). The line between the homologous antigen and antisera appeared earlier, i. e. in about 18 hours, and that between measles serum and distemper antigen appeared but faintly rather later (in 24 hours time); the two lines joined within the next 48 to 72 hours. However, when this experiment was repeated with measles infected ferret lung (572) and measles antiserum no lines were seen. This was attributed either to the low concentration or the absence of the measles antigen in the lung tissue used.



White and Cowan (1962) have shown with rinderpest virus antigens consisting of tissue culture fluids or infected tissues of the same high infectivity titre, precipitation lines were seen with the tissue antigens; but not with the tissue culture fluid. When however the tissue culture fluid had been concentrated a hundred fold by precipitation with ammonium sulphate precipitation lines were obtained. This finding suggested to them that the viral antigen which had been precipitated was a protein.

Early in the course of this work in Spring of 1961, attempts were made to obtain a diffusion lines using the measles virus as the antigen. The virus was grown in rapidly multiplying continuous cell lines to get a yield of high titre virus in tissue culture, and also in the slow growing primary amnion cells (Monkey kidney cells were precluded on account of the foamy agents). The virus was concentrated as described before (page 95 ). With HEP-2 cells measles antigens of high infective titre were obtained but with lower complement fixing and haemagglutinating properties whereas the virus grown in primary amnion cells had a lower infective titre, but with comparatively higher complement fixing and haemagglutinating properties (see table 195 ). This difference may be explained by the cell

systems used. The ratio of cell associated virus (CAV) to the released virus (RV) ( $\frac{\text{CAV}}{\text{RV}}$ ) being almost one with human amnion cells, and greater than one with HEP-2 cells grown virus. The former has a greater content of both CAV and RV (Franklin 1962) and the CAV may be better protected and thus more thermostable than the released virus. This protective effect may be associated with the "outer membrane" described by Baker et al (1960) in their electron microscopic studies of amnion grown measles virus.

The tube methods have the advantage over the macro and micro-methods in that the lines of precipitation are sharper and more clear cut; observation is thus easier due perhaps to the better optical qualities that pertain. Furthermore the individual fractions in a complex system of this type were precipitated more obviously and lines which were barely visible using other techniques, were clearly seen. In this tube method (see table of results page 208-9) two diffuse lines were seen between HEP-2 grown measles antigen and antisera, and one diffusion line between measles antigen and distemper antisera. Both antisera gave a single precipitation line with distemper antigen.

Using the tube method it was found that there were three bands of precipitation which appeared when the amnion grown measles virus was set up against its homologous antiserum. The third of these bands was very slow to appear and was detected only three weeks after setting up the test.

Thus, it would appear possible that there are three antigenic components contained in the measles virus. In similar experiments with distemper virus and its own antiserum only two antigenic components were revealed by this method. When either virus was allowed to diffuse against the heterologous serum (i. e. measles against distemper antiserum, or distemper against measles antiserum) only two bands of precipitation were detected.

The above factors may have a bearing on the structure of measles virus grown in different tissue systems. The complete particle, it can be assumed, contains, as the basic unit, infective, complement fixing and haemagglutinating properties. In the rapidly dividing HEP-2 cells the measles virus attains high infective titres but the process of maturation of the new virus seems to have insufficient time to reach completion because only very small amounts of C. F. antigen and haemagglutinin are produced. In the slower growing

amnion cells C. F. antigen is produced in much greater amounts as also is the haemagglutinin. The fact that the infectivity titre of measles virus cultured in this way is much lower than in HEP-2 propagated virus can be attributed partly to the prolonged effects of thermal inactivation and partly to slower multiplication of, and the smaller numbers of amnion cells.

The high yield of infective virus is probably a reflection of the rapid multiplication of the HEP-2 cells. Roizman and Shøfluederberg (1961) have demonstrated by fluorescent labelled staining of HEP-2 cell cultures infected with measles virus, that the virus readily infects and multiplies in the dividing cells and that these cells become involved in the formation of syncytia.

Another point to note is the amount of cytoplasm in each of these cell systems. Uninfected amniotic cell cultures as compared with uninfected HEP-2 cells have a larger amount of cytoplasm, and the nuclei contain evenly distributed chromatin with more intensely basophilic nucleoli. In their growth, too, the rapidly dividing HEP-2 cells metabolise quicker - hence the higher buffering and more frequent changes (every third day) can be maintained with maintenance medium changed much less frequently (i. e.



weekly).

Thus the measles virus may contain three antigenic components, one more than the distemper virus. This finding was confirmed by the experiments described using the immune electrophoresis method. These results are in accordance with those obtained by Schluederberg and Roizman (1961) who, using cesium chloride density gradient methods, were able to separate three fractions, in the case of measles and Cowan and White (1962) who found two fractions in distemper.

### Experimental Infection

In experimental animals too the behaviour of these two viruses are very dissimilar. It has been shown in this work that in the adult ferrets, distemper has a lethal action, and with the measles virus no effect was seen.

An attempt was made to see whether suckling ferrets could be infected with measles virus (page 174. ). Cortisone was used as this would help in the spread of the virus by keeping down the protective inflammatory mechanism, and when used over prolonged periods would have a deleterious effect on the lymphoid tissues, thus adversely affecting the antibody formation. In addition it also causes an involution of bone marrow. When ferrets were inoculated by the intra-cerebral and intra-peritoneal routes the lesions noticed were hydrocephalus (? encephalitis) paraplegia and fever. The latter febrile response could definitely be attributed to measles infection helped by cortisone. Whether the other two effects are attributable to the virus, or to the trauma caused is very difficult to judge. However, measles virus was recovered from the brain (page 178. ).

When guinea pigs were inoculated subcutaneously and intra-peritoneally with the ferret kidney <sup>grown</sup> distemper virus ~~grown~~ caused a marked loss of weight, with staring <sup>rough</sup> fur.

No such reaction was observed with viable measles virus.

In mice, according to Carlstrom (1958), the symptoms of measles infected mice are indistinguishable from those of canine distemper. However, in this work the experience was different. When attempting to repeat Carlstrom's work it was found that mice at an age of 3 days were resistant to the effects of the measles virus and that even at an age of 24 hours, no constant effect was observed. When mice, were inoculated intra-cerebrally at the age of less than 12 hours they developed a spastic paraplegia and died in 5 to 7 days; within 3 to 4 passages this effect was constantly and regularly observed and the measles virus had become adapted to the new host. It is unlikely that this could have been a traumatic effect because it occurred as long as 7 days after inoculation and because no sign of haemorrhage was found in the brain at autopsy.

A sequence of events contrasting strongly with those described occurred when mice were inoculated with a wild strain of distemper virus. Firstly, 4 - 5 days old mice were completely susceptible and within 3 - 5 days they became hyperactive and developed convulsions and died rapidly from encephalitis. Thus the effect was much shorter in duration and had a more dramatic encephalitis. Passage from the

infected mouse brains resulted in the regular killing of all inoculated animals in the first and all subsequent attempts.

With the avian tissues the difference in the behaviour of the two viruses is also marked. Attempts to grow the measles virus in eggs have continued from the days of Rake and Sheaffer (1940) and Enders (1940). These were suspended because the only definite indications of the virus growth were infection in man or monkey. With the advent of "in vitro" markers in tissue cultures, the growth of the virus was, however, more easily established., (Enders 1956), Milanovic et al (1957), Katz et al (1958), Growth of the virus in chick embryo tissue culture was easily achieved from an egg adapted strain, but the virus lacked the haemagglutination and the complement fixing properties were very much diminished, whilst infectivity was retained.

Compared with the above, the distemper virus was easily adapted to growth on the chorio-allantoic membrane but it became attenuated. It is to be noted that on adaptation to the egg, the Lederle and the Onderstepoort strain behave slightly differently. The Lederle strain (Cabasso, 1959) does not produce any cytopathic effect in chick embryo tissue culture, whereas the Onderstepoort strain (Karzon &



Bussel 1959) produced a cytopathic effect which consisted of degeneration of cells. No giant cell or syncytial formation resembling that of egg adapted measles strain is seen.

### Antibody responses

When the natural infection with measles occurs in the human being there is a sharp rise in the titre of homologous C. F. antibody, which appears in about the 8th - 10th day but at this time C. F. antibody against the distemper virus was not detected in five out of six paired serum samples. In the sixth serum complement fixing antibodies for distemper appeared at the 21st day. In Beck's cases (1960) the complement fixing antibodies for distemper appeared on the 9th day in one patient who was 10 years old in other patients aged 20 - 40 years much earlier - on the 2nd and 6th days. In my case, too, the child who developed both antibodies was a 12 year old boy. From this one can speculate that the age of the individual patient may influence the development of the antibodies for both measles and distemper virus, a finding borne out by the distemper neutralising antibody titres found in human sera by other workers (Karzon 1955, Carlstrom 1956, Hopper 1959)<sup>b</sup>. In my series of cases, however, it was not possible to follow up the cases and obtain repeated samples of serum because the patients were usually discharged from the hospital on or about the 10th day and did not return subsequently.

The study of development of antibodies in clinical

cases of distemper was virtually impossible, because samples of blood in the first few days of the acute phase of the illness could never be obtained. In the experimental ferret serum samples taken in the acute phase could be obtained, but since the infection was invariably fatal, convalescent sera could not be obtained.

Thus the only series of sera obtained from ferrets infected with distemper virus came from animals immunised with the virulent EPIVAX strain and subsequently challenged with a virulent wild strain. In such animals the complement fixing antibodies were estimated ten days after the last injection but measles antibodies were not detected at dilutions of 1 in 2, in three out of the four animals, and in the fourth a year old hob ferret only a titre of 1:4 was detected.

A similar finding was obtained in guinea pigs when the virulent ferret kidney grown distemper virus was used for immunisation. Only distemper complement fixing antibodies were developed and none active against measles was detected.

Thus in the natural infection of measles and in infection and immunisation in the laboratory animals with distemper, high antibody titres to the homologous virus are formed more rapidly (in about 10 days or less) and

antibodies to the heterologous virus are either absent or develop to a low level after a further lapse of seven or more days. Again it is interesting that in measles immunised ferrets the only animal to develop heterologous antibody (to distemper) was much older than those that did not.

A human anti-measles serum, with a titre of 1:256 by complement fixation tests, failed completely to neutralise a wild strain of distemper virus and when the virus serum mixtures were inoculated into two ferrets, both developed typical and fatal distemper, but after a prolonged incubation period. This result is in conformity with that of other workers (Adam et al 1957).

Seven ferrets which received a series of immunising inoculations with measles virus developed complement fixing antibodies to a high titre (see page 189). When three of these animals were challenged, after an interval of four weeks, they developed typical distemper and died after a prolonged incubation period., i.e. on the 18th and 19th day, 8 to 9 days later than the control animals. Four further animals were challenged after 10 weeks and died after 10 - 14 days an interval which did not appreciably differ from the control animals.

Thus the animal neutralisation tests indicate that



the protection against distemper afforded by previous measles vaccination with the measles virus is minimal and in this work was only indicated by prolongation of the incubation period. However, it is to be appreciated that the distemper virus is highly infective and virulent for ferrets, whereas the measles virus does not cause any disease in that species.

To obviate these difficulties Carlstrom (1958)<sub>b</sub> used suckling mice - and mouse brain adapted measles and distemper virus strains. Using the minimal viral dosage to give a hundred per cent kill with both viruses and using 24 and 17 day old human convalescent measles serum, rabbit hyperimmune measles serum, a dog convalescent distemper, and rabbit hyperimmune distemper serum, he performed cross-protection tests. He concluded that the neutralisation tests were more specific than C. F. tests, and that there was an antigenic relationship between the two viruses; the difference was that the measles serum neutralised the homologous antigen to a greater extent, than the heterologous antigen, and that the distemper serum, neutralised both the antigens to an equal extent. In this work using tissue culture cells and embryonated eggs (pages 194. ) it was found that a cross protection did exist, but the results different from

Carlstrom's experiments in that in both cases the homologous neutralisation was higher than the heterologous; it was particularly well marked in the case of measles.

The measles, distemper, rinderpest group of viruses has, as a group, many resemblances to the herpes simplex, virus B, pseudorabies group. Each group of three viruses contains one member highly pathogenic for a particular animal host, rinderpest in the measles group and virus B. in the herpes group. Each also contains one member highly specialised for the human host, measles in the former, herpes simplex in the latter group. Within these groups the members show a considerable amount of antigenic sharing although the crossing may be in one direction only. Thus, virus B. seems to have a more complete antigenic structure than herpes simplex because an antiserum against itself neutralises herpes simplex as well, or better than, it neutralises itself. A herpes simplex antiserum, however, has but little power to neutralise virus B. Such a state of affairs might well exist in the measles group. This hypothesis would be supported by the fact that the protection afforded by a measles antiserum to ferrets challenged with distemper virus was of a very low order or absent. Studies of the protective value of a rinderpest antiserum in protecting animals against the three viruses of the measles group would clarify this position but in this country it is not possible because of the strict pro-

hibition of the importation of strains of the rinderpest virus.

It has been said (Burnet 1959) that in a long established parasite host relationships, the most successful is the one where the parasite will have the optimum virulence for the particular host. The parasite should cause a mild disease with a low death rate - a mild rather long lasting infection, in which both survive (Andrewes 1960). The other requirements are, that there should be no serious damage to the host and that there should be adequate opportunity for the transfer of the parasite to fresh hosts - the result of mutual adaptation. Such a balance is seen between measles and the human host.

When the parasite invades a new host species the result is unpredictable and depends upon many factors. If the parasite finds the new environment unsuitable it may perish, unable to synthesise the nourishment it needs, and or to cause infection. The host cell, too, may undergo certain changes. Due to prolonged contact with the virus, the defence mechanism of the host cell may be altered and according to Andrews (1960) "may itself change through the operation of genetic or immunological factors". Similarly, the parasite may be affected, and in the end a stable balance



will be the results.

Thus a parasite after prolonged contact with an originally refractory host may establish a relationship, and thence be transferred to infect a new host species, which had no acquired or racial immunity. In such a host it causes a high mortality, with repeated transfer of infection over a period of years.

The dog has been in close contact with man from pre-historic times, probably the first animal to be domesticated by man. Thus it has received repeated doses of measles virus, perhaps over thousands of years. In this new species of host, a more severe disease has resulted and a stable balance is yet to be achieved. The virus too undergoes certain changes - the mutant retaining some original properties such as the infectivity, partly losing some (e.g. complement fixation and completely losing others as for example haemagglutination. It may also develop certain new properties like the extensive invasive properties which may be the origin of the severe complications described in distemper.

The Rinderpest virus belongs to the same group of viruses as measles and distemper. It may have pursued a different course of evolution in its bovine host to produce this time a highly virulent and lethal infection.

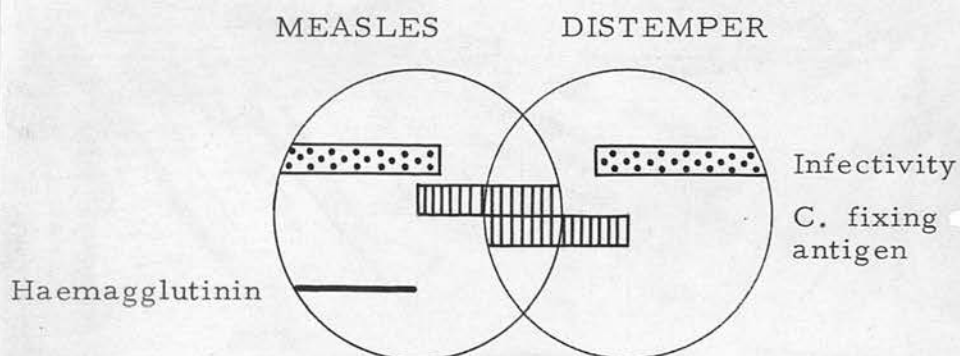


Diagram to show the antigenic overlapping in measles and distemper viruses.

When the distemper and ~~measles~~<sup>vindepest</sup> viruses are as perfectly adapted to their hosts as the measles virus is to man, they will no longer be able to kill or cause so much distress.

Once the parasite has adapted to a new host, it is at first virulent to the new host and at the same time loses its virulence or becomes attenuated for its original host. Thus, one can explain the mild or complete lack of reaction when the avianised canine distemper was used as vaccine for measles infection. (Adams et al, 1959, Carlstrom 1959, Hoekenga, 1960).

"There is a close correspondence between the optimal temperature for growth and the sensitivity of the virus for interferon - the higher the optimum temperature the less sensitive was the virus to interferon". (Isaacs 1962). It may well be that the marked differences in the severity, duration and recovery times in measles and distemper are a reflection on the amount of interferon which the viruses cause the host to produce in their natural hosts. It is known that the measles virus produces interferon and that attenuated strains give rise to more than the wild and more virulent strains (Enders 1960). No evidence has yet been produced that the distemper virus can produce interferon.

The normal temperatures of the two natural hosts are different - in man it is  $98.4^{\circ}\text{F}$  and in puppies  $102.6^{\circ}\text{F}$  (Paterson 1957). The temperature for optimal growth for the measles virus is  $37^{\circ}\text{C}$  ( $98.4^{\circ}\text{F}$ ) and as shown in this work for the distemper virus it is  $39^{\circ}\text{C}$ .

The time of onset and the height of the fever in these two infections are factors which are known profoundly to influence the course of the illnesses. In measles the sharp rise in fever after four days of prodromal illness occurs at a time when there has been an opportunity for the cells to produce a fair yield of interferon and the elevated temperature is in itself a factor which would directly inactivate the virus (Black et al 1959). Thus the viraemic phase is short lived and the virus has not been recovered from the patient's blood after the first day of the rash (see page III ). When a virus accustomed to grow at  $37^{\circ}\text{C}$  or at a lower temperature is suddenly confronted with a higher temperature it may, especially in the cases of avirulent strains, stimulate a higher level of interferon production. Thus, within a few days of the onset of the rash and fever, the average case of measles begins to make an uneventful recovery.

The rise in temperature, however, has quite a different effect in distemper because the virus has a much



higher optimal growth temperature. Such a virus is able to cause only small amounts of interferon production or, indeed, no interferon at all may appear in the host cells. Thus the raised temperature and continued fever in distemper is accompanied by a severe and toxic illness, widespread viral involvement of the tissues, a prolonged course, and secondary bacterial infection. These factors may also account for the virulence of the distemper virus as is manifested by its ease of adaptation to a wide variety of hosts e.g., the suckling mouse (page 183 ) and many genera of the sub-order Canidae and Mustelidae etc.

Throughout all the observations made in this thesis and during the survey of literature it has become increasingly apparent that the measles and distemper group of viruses have more properties in common with the herpes group of viruses than with the larger myxoviruses. The one outstanding difference between the two groups is that the measles group have a helical symmetry while the herpes virus capsids have a cubical arrangement. The following table has been introduced to show these resemblances and differences. The measles group of viruses by virtue of their characteristic cytopathic effect and syncytium formation their intra nuclear inclusions, and their D.N.A. content seem closer to the herpes viruses; the properties shared by the group with the Newcastle and mumps viruses are the helical component and haemagglutination. This view is subscribed to in Enders' (1954) classification based on the type of cytopathic effect produced, and is in accord with Cooper's classification (1962).

	NDV + Mumps	MV	CDV	HERPES
Particle size	100-230	120-140	90-140	110
<u>Election microscopic studies</u>				
Symmetry	helical	helical	helical	cubical
Membrane		present	present	present
<u>Cytopathic effect</u>				
Syncytium	+	+	+	+
<u>Inclusion bodies</u>				
Intra-nuclear (Type A)	-	+	+	+
Cytoplasmic	-	+	+	+
Haemagglutinin	+++	+	-	-
<u>Elution of the virus</u>	+	-	-	-
Haemolysin	++	+	-	+
Interferon	+++	+	?	?
A. O. stain	RN <sup>+</sup>	DNA	DNA	DNA
Neuraminidase	+	-	-	-
<u>Principal host</u>	chick & man	Man	Dogs	Man
Tissue prelediction	Rsp. (Neuo)	Epi	Epi & Endo	Epi
Soluble antigen	+	-	-	-
Ether sensitivity	+	+	+	+

Rsp. = Respiratory

Neuo = Neurotropic

Epi = Epithelial

Endo = Endothelial

### SUMMARY

1. A virological study of 15 children below the age of 12 years, suffering from measles diagnosed by the senior medical staff of the City Fever Hospital in Edinburgh was undertaken. The measles virus was isolated from the blood of five, cases, from throat swabs of four, from the urine of one, and from the lung of a fatal case of measles meningo-encephalitis. Neutralisation and complement fixation methods were used to estimate the antibody levels to both viruses. The titres of complement fixing antibodies to measles ranged from 1 in 8 to 1 in 512 but those for the distemper virus were appreciably lower varying between 0 and 1 in 32.
2. A somewhat similar study was made on dogs seen in the Small Animal Clinic at the Royal Dick Veterinary College. Virulent strains of the distemper virus were isolated from the blood and spleen of one adult dog and three puppies found suffering from distemper and later destroyed. Antibody levels were estimated from six dogs in the late stages of infection,—animals which were later destroyed. Complement fixing antibody levels for the distemper virus ranged from 1 in 8 to 1 in 128 but for the measles virus none were higher than 1 in 8.
3. By using a combination of pancreatin and trypsin



instead of trypsin alone it was found that the yield of cells for culture from human amniotic membrane was increased at least one and a half fold and that this technique was so satisfactory that it was employed throughout.

4. A study of the cytopathic effects of the measles virus was made in human amnion and human kidney primary tissue cultures and in secondary monkey kidney cell cultures and was extended to include the following human cell lines:- HeLa, HEP-2, and a local strain of foetal lung cells. The measles virus also grew well in primary cultures of ferret kidney producing typical giant cells.

5. A comparative study using various staining methods was made on the morphology of the giant cells, syncytia, and inclusion bodies produced by the two viruses in tissue culture. The measles virus produced the larger syncytia with nuclei scattered evenly throughout while the distemper virus produced a rather large giant cell with a cluster of nuclei at the centre and also with a peripheral ring of nuclei. "Stellate" cells were more numerous in measles cultures than in distemper infected cells. Little or no difference in the nature or the site of the inclusion bodies could be made out between the two viruses.

6. The distemper virus could not be adapted to grow in

primary cultures of human amnion or secondary monkey kidney cells: nor was it found possible to adapt the virus to grow in cell lines of human origin, e.g., HeLa, HEP-2, or foetal lung.

7. The distemper virus grew satisfactorily in ferret or dog kidney tissue cultures. Growth of the virus in ferret kidney cells was more rapid and the cytopathic effect more profound when the cultures were incubated at 39°C instead of 37°C.

8. A study of the morbid anatomy of the ferret infected with virulent distemper virus was made and included a histopathological comparison of the tissues of normal and diseased animals. The lesions were more severe than those described for measles in man but the giant cells in the lymphoid tissues were practically identical with the Warthin-Finkeldey cells in human tissues.

9. Both measles and distemper viruses were adapted to grow in suckling mice in which host a spastic paralysis and death occurred.

10. An attempt to infect adult and young six week old ferrets with the measles virus failed. Infection, however, was achieved when the virus was injected intra-cerebrally into two-day old suckling ferrets under the influence of cortisone. In one animal the virus was recovered ten weeks

after inoculation.

11. When ferrets were immunised with the measles virus they were not protected against a lethal challenge by a virulent strain of the distemper virus. However, there was a slight prolongation of the incubation period.

12. When ferrets were immunised with attenuated distemper virus followed later by an injection of virulent virus they developed high levels of antibodies to distemper but only low levels to the measles virus.

13. The measles virus was found to be able to agglutinate the red blood cells of rhesus monkeys and the serum of convalescent measles patients had a power to inhibit this haemagglutination. The complement fixing, neutralising, and haemagglutination inhibiting properties of sera from measles patients ran parallel.

14. The canine distemper virus was found to possess no haemagglutinating property. Canine sera with high levels of antibody to the distemper virus did not inhibit haemagglutination by the measles virus

15. A comparison of the serologic findings in measles and distemper was made using a wide variety of immunological techniques including neutralisation tests in animals, eggs and tissue cultures as well as complement fixation and

agar gel diffusion. It was apparent in complement fixation and in some neutralisation experiments that a moderate degree of antigenic sharing was present but infection with one virus did little to protect an animal against infection with the other.

16. In agar gel diffusion experiments the measles virus gave three bands of precipitation when it was set up against its homologous serum whereas the distemper virus gave only two. This finding, which was confirmed by immune electrophoresis is interpreted as indicating that the measles virus possesses an antigen not present in the distemper virus.

This antigen may possibly be the haemagglutinin.

17. It was found that 5 fluoro-deoxyuridine inhibited the growth of the measles virus in infected HeLa and monkey kidney cells. Since this substance did not influence the multiplication of the poliomyelitis virus type 1 a known ribo virus and did inhibit the growth of the vaccinia virus a known deoxyvirus it is concluded that the measles viruses contain deoxyribonucleic acid.

18. Further evidence that the measles and distemper viruses may be a deoxyvirus was provided by preparations which had been stained with the fluorescent dye acridine orange. Infected cells, giant cells and syncytia stained with



an apple green D. N. A. fluorescence throughout and the red R. N. A. staining property of the normal cell was lost. A similar colouration was seen in the viral inclusions in the nucleus and in the cytoplasm.

19. Electron-microscopical examination of the two viruses by negative staining methods shows them to be almost identical, the only difference between the two being the thicker limiting membrane of the measles virus.

20. Both viruses possess a helical symmetry resembling somewhat that of the Newcastle and mumps viruses.

21. When the particles of both viruses have been disrupted disc like structures as well as the helix are liberated. It is suggested that the discs are capsomeres (sub units) which are attached to a central spiral core of deoxyribonucleic acid and that as they protrude they confer on it a typical "herring bone" appearance.

22. The position of the measles, distemper, rinderpest group of viruses in relation to the herpes group and other viruses is discussed.

APPENDIX

Table showing the comparative stability of measles and canine distemper virus at

different temperatures:

<u>MEASLES</u>			
<u>Temperature</u>	<u>Loss in Infectivity</u>	<u>Time</u>	<u>Authors</u>
-72°	0.4 log units	36 mths. (with 5% sera)	Musser & Underwood (1962)
	1.1 log units	do (without sera)	Underwood (1959)
-70°	2.8 TCID/50 ml.	18 weeks	Warren (1960)
-30°C	1.4 log TCID (with sera)		
	3.1 log TCID/50 ml. (without sera)	36 mths.	Musser & Underwood
-15 to 20°C	3 log units	60 days	Girardi & Warren (1958)
+6°C	2.8 log units (with serum) complete without sera	54 hours	Musser & Underwood (1960)
+22°C	0.9 log units	4 days	Ruckle & Rogers (1957)
+23°C	1.0 log unit	1 day	Black (1959)
+35°C	1.2 log unit	1 day	Muelturing et al (1957)
+36.5°C	1.2 log unit	1 day	Ruckle & Rogers (1957)
+37°C	2 log units	1 day	Muelturing et al (1957)
+37°C	3.2 log units	1 day	Black, Reisin & Muelturing (1959)
37°C (pH 7.5)	0.15 log units per hr. 0.3		Black (1959)
56°C	Complete	30 mins.	
56°C	Complete	1 day	Girardi & Warren (1958)
<u>DISTEMPER</u>			
-24°C	Remained infective	over 2 yrs.	Sideroff & Green (1942)
+25°C	Complete	8 days	Celiker & Gillespie (1954)
+32°C	More than 3 log units	48 hours	Haig (1956)
+37°C	Remained infective	3 - 6 days	Laidlaw & Dunkin (1926)
+50°C	Complete	1 hour	Celiker & Gillespie

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Robinson, M., Roberts, J. L., & ...  
Owen, E. M., ...  
Melnick, J. L.

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Black, F. L.

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## BIBLIOGRAPHY

Abbreviations

The abbreviations used in this bibliography  
are those listed in "World Medical Periodical"  
by the World Medical Association, 1962.

Bryan, J.

Budd

Burke & ...

Burke & ...

Burns, F. M.



Please note that Pages 1, 2, 3 have been transposed  
by the binder.

- Beynesh, M., Pollard, E. C. 1958 Virology., 5, 256.  
 Opton, E. M., Black, F. L. & Melnick, J. L.
- Bindrich, H. 1954 Arch. exp. Vet. Med., 8, 231.
- Black, F. L. 1958 J. Immunol., 83, 74.
- Black, F. L. 1959 Virology, 1, 184.
- Black, F. L., Reissig, M., & Melnick, J. L. 1956 Proc. Soc. exp. Biol. (N. Y.), 93, 107.
- Black, F. L., Reissig, M. & Melnick, J. L. 1959 Advanc. Virus Res. 6, 205.
- Blaine 1832 Canine Pathology, 3rd Edn. T. & T. Boosey, Lond. p. 97.
- Blake F. G., & Trask, J. D. 1921 J. exp. Med. 33, 385
- Bolande, R. P. 1961 New Eng. J. Med., 265 919.
- Bosch, L., Harbers E. & Heidelberger, C. 1958 Cancer Res. 18, 335.
- Brødstreet, C. M. P. & Taylor, C. E. D. 1962 Monthly Bull. Minist. Hlth. Lab. Serv. 21, 96
- Brenner, S. & Horne R. W. 1959 Biochem. biophys. Acta., 34, 103.
- Bryan, H., 1928 Ved. Med. 23, 296.
- Budd 1859
- Burke & Issacs, A. 1957a Brit. J. exp. Path. 39, 78.
- Burke & Issacs, A. 1957b IBID 39, 452.
- Burnet, F. M. 1959 Principles of Animal Virology, Academic Press

- Arakawa, S., Muto, S., 1959 Yokohama, Med. J.,  
Kaneko, T. & Seki, T., 10, 179.
- Armstrong, J.A., 1956 Exp. Cell. Res. 11, 640.
- " .., & Niven, 1957 Nature (Lond.) 180, 1335  
J.S.F.,
- Ayerbach, R., Grobstein, C. 1958 Exp. Cell. Res. 15, 384.
- Babbot, F.L., and Gordon, J.E. 1954 Amer. J. med. Sci.,  
228, 334.
- Baker, R.F. Gordon, I., & 1960 Nature (Lond), 185, 790  
Rapp, F.,
- Bateman, J.K. 1954 Vet. Rec. 66, 343.
- Bech, V. 1959 J. Immunol. 83, 267.
- Bech, V. 1960a Acta. path. microbiol.  
scand. 50, 331.
- Bech, V. 1960b IBID 322.
- Bech, V. 1962 Amer. J. Dis. Child,  
103, 252.
- Bech, V., & Von Magnus 1957 Acta. path. microbiol.  
scand. 42, 1.
- Bech, V., & Von Magnus 1958 IBID 42, 75.
- Becker 1957
- Bedson, S.P., & Bland, J.O.W. 1932 Brit. J. exp. Path. 13,  
461.
- Berger, E. & Melnick, J.L. 1962 Progress in Medical  
Virology Karger/Basel,  
4, 29.
- Beveridge, N.I. & Burnet, F.M. 1946 M.R.C. spec. Rep.  
No. 256 (HMSO)

Ackerman, P.H. & Black, F.L.,	1961	Proc. nat. Acad. Sci. (Wash), <u>47</u> , 213.
Adams, J.M.,	1941	J. Amer. med. Ass., <u>116</u> , 925.
" ..	1948	IBID <u>138</u> , 1142.
" ..	1953	Pediatrics., <u>11</u> , 15.
" .., Green R.G., Evans, C.A., and Beach N.,	1942	J. Pediat, <u>20</u> , 405.
Adams, J.M., & Imagawa,D.T.	1957a	Pediat. Clin. N. Amer. P. 193.
" .. "	1957b	Proc. Soc. exp. Biol. (N.Y.), <u>96</u> , 240.
" .. " Chadwick, D.L., Gates, and Yoshimori, M.S.,	1957	Amer. J. Disc. Child <u>94</u> , 409.
Adams., J.M., Imagawa,D.T. Chadwick, D.L., Gates., and Iem, R.A.	1958	IBID, <u>95</u> , 601
Adams, J.M. Imagawa,D.T. Wright, J.W., & Tarzon,G.	1959	Virology, <u>1</u> , 351.
Adams, J.M., Imagawa, D.T. Yoshimori, M., & Huntington, R.N.,	1956	Pediatrics, <u>18</u> , 888
Alagna, G.	1911	Arch. Laryngol. Rhinol, <u>25</u> , 527.
Anderson, J.F., & Goldberger	1911	Publ Hlth. Rep (Wash), <u>26</u> , 887
Arakawa, S. Hoshino, J, Kaneko, T., & Seki, T.	1959	Arch. ges. Virusforsch, <u>9</u> , 656.
Arakawa, S. Hoshino, J. Nagashima, H. Kameko T., & Seki, T.	1959	IBID, <u>9</u> , 674.



4

Burnet, F.M.	1962	Natural history of infectious diseases C. U. P.
Bussel, R. H.	1960	Fed. Proc. <u>19</u> , 1.
Cabasso, V. J.	1951	Vet. Med. <u>46</u> , 167.
Cabasso, V. J.	1955	Proc. Soc. exp. Biol. (N. Y.) <u>88</u> , 199.
Cabasso, & Cox, H. R.	1949	IBID <u>71</u> , 246.
Cabasso, Kiser, K., & Stebbins, M. R.	1959	IBID <u>100</u> , 551.
Cabasso, V. J., Avampato, Kiser, K., & Stebbins, M. R.	1960	IBID <u>104</u> , 526.
Carlstrom, G.	1956	Acta. pediat. (Uppsala), <u>45</u> , 180.
Carlstrom, G.	1957	Lancet, <u>273</u> , 344.
Carlstrom, G.	1958a	Arch. ges Virusforsch. <u>8</u> , 539.
Carlstrom, G.	1958b	IBID <u>8</u> , 584.
Carlstrom, G.	1959	Acta. pediat. (Uppsala) <u>48</u> , 25.
Carre, H.	1905	C. R. Acad. Sci. (Paris) <u>140</u> , 689.
Carre, H.	1926	Rev. gen. Med. Vet. <u>35</u> , 545.
Count, A. E., Randle, C. J. M. & Downie, A. W.	1961	J. Hyg. Camb. <u>59</u> , 249.
Celliker & Gillespie	1954	Cornell. Vet., <u>44</u> , 276.
Christensen, P. E., Schmidt, H., Jensen, O., Bang, H. O. Anderson, V. & Jordan, B.	1952	Acta. med. scand., <u>144</u> , 32.

- Clarke, D.H. & Casals, J. 1958 Amer. J. trop. Med. Hyg. 1, 561.
- Coffin, D. L. 1959 Ann. N.Y. Acad. Sci. 81, 164.
- Coffin, D. L., & Chien, L. (1957) Virology, 3, 132.
- Cohen, S.M., Gordon, I, (1955) Proc. Soc. exp. Biol. (N.Y.) 90, 118.  
Rapp, F, Macaulay, J.C. & Buckley, S.M.
- Cooper P.D. 1961 Nature (Lond) 190, 302.
- Copeman 1900 Proc. Roy. Soc. 67, 459.
- Corbett, E. U. 1945 Amer. J. Path. 21, 905.
- Crowle, A. T. 1960 Int. Arch. Allergy. 16, 13.
- Cruickshank, R. 1961 Lister fellowship lecture p. 25.
- Cruickshank, J.G., Waterson, 1962 Res. vet. Sci. 3, 485.  
A.P.; Kanarek, A.D. & Berry, D.M.
- Cutchins, E. C. & Dayhuff, T.D. 1962 Virology 17, 420.
- Dedie, K. & Klapotke, K. 1951 Exp. Vet. Med., 4, 137.
- Dedie, K. & Klapotke, K. 1952 Vet. Bull., 23, 40.
- Dekking, F. & McCarthy K. 1956 Proc. Soc. exp. Biol. (N.Y.) 93, 1.
- De. Meyer, E. 1960 Virology, 11, 634.
- De. Meyer, E. & Enders, J.F. 1961 Proc. Soc. exp. Biol. (N.Y.) 107, 573.
- Diagnostic procedures 1956 Diagnostic procedures for Viral & Rickettsial disease.

- Duale & D'Aunoy 1922 J. exp. Med., 36, 391.
- Dulbecco, R. & Vogt. M. 1954 IBID, 99, 167.
- Duncan, I. B. R. & Bell, E. J. 1961 Brit. med. J. 2, 866.
- Dunkin, G. W. & Laidlaw, P. P. 1926 J. comp. Path., 39, 201.
- Dunkin G. W. & Laidlaw, P. P. 1934
- Elkin, I. I. 1961 A Course in Epidemiology Pargamon Press, Lond. p. 355.
- Enders, J. F. 1940 Virus and Rickettsial diseases, a symposium held at the Harvard School of Public Health. (1939) Harvard. Uni. Press. p. 237.
- Enders, J. F. 1954 Ann. Rev. Microbiol. 8, 473.
- Enders, J. F. 1956a Ann. intern. Med. 45, 331.
- Enders, J. F. 1956b Amer. J. med. Sci., 231, 622.
- Enders, J. F. 1960 Trans. Coll. Phycns. Philad., 28, 68.
- Enders, J. F., Katz, S. L., Milanovic, M. V. & Halloway, A. 1960 New Engl. J. Med., 263, 153.
- Enders, J. F., McCarthy, K. 1959 IBID 261, 875.
- Mitels, A., & Cheatham, W. J.
- Enders, J. F. & Peebles, T. C. 1954 Proc. Soc. exp. Biol. (N. Y.), 86, 272.

- 7
- Enders, J. F., Peebles, T. C. 1957 Amer. J. publ. Hlth.  
 McCarthy, K. Milanovic, M. V. 47, 275  
 Mitus, A., & Halloway, A.
- Ferguson, J. & Tobri, J. O. H. 1958 Brit. med. J. 5053
- Ferry, N. S. 1911 J. infect. Dis., 8, 399.
- Finkeldey, N. 1931 Arch. path. Anat.,  
281, 323.
- Frankel, J. W. 1958 Fed. Proc. 17, 511.
- Frankel, J. W., Burnstein T. 1957 Bact. Proc. p. 76  
 Deviney, J. T.
- Frankel, J. W., Burnstein T. 1958 Fed. Proc. 17, 511.  
 & West, M. K.
- Frankel, Cooke, H., Deviney, 1958 Bact. Proc. p. 58  
 J. T., Warner, M., & West,  
 M. K.
- Frankel, J. W., & West M. K. 1958 Proc. Soc. exp. Biol.  
 (N. Y.), 97, 741.
- Franklin, R. M. 1962 Progress in Medical  
 Virology Karger/Basle  
4, 1.
- Gibbs, F. A., Gibbs, E. L.,  
 Carpenter, P. R., & Spies, H. N. 1959 J. Amer. med. Ass.  
171, 1050.
- Gillespie, J. H. 1959 N. Amer. Vet. 32,  
 837.
- Gillespie, J. H. & Karzon, D. T. 1960 Proc. Soc. exp. Biol  
 (N. Y.) 105, 547.
- Girardi, A. J. 1957 Fed. Proc. 16, 414.
- Girardi, A. J. & Warren, J. 1958 Fed. Proc. 17, 513.
- Goffe, A. P. 1962 Amer. J. Dis child,  
103, 327.



Goldberger, J. & Anderson, J. F.	1911	J. Amer. med. Ass. <u>57</u> , 971.
Goldfield, M., Boyer, N. H., & Weinstein, L.	1955	J. Pediat., <u>46</u> , 30.
Grasset, E., Bonifas, V., & Pongratz	1958	Proc. Soc. exp. Biol. (N. Y.), <u>97</u> , 72.
Gray, A. R.	1961	Immunology, <u>4</u> , 253.
Green, R. G.	1939	J. Amer. vet med. Ass. <u>95</u> , 465.
Green, R. G. & Carlson, W. E.	1945	IBID <u>107</u> , 131.
Gresser, I., & Katz, S. L.	1960	New Engl. J. Med., <u>263</u> , 452.
Grist, N. R.	1950	Glasg. med. J. <u>31</u> , 431
Grund	1922	J. infect. Dis. <u>30</u> , 86.
Gutiera, J. C. & Gorham, J. R.	1955	Amer. J. vet. Res. <u>16</u> , 325.
Hagan, W. A. & Brunner, D. S.	1957	Infectious diseases of domestic animals. 3rd Edn. Baillier. Tindall & Cox., p. 791
Haig, D. A.	1948	Onderstepoort, J. Vet. <u>23</u> , 149.
Haig, D. A.	1958	J. South Afr. vet. med. Ass. <u>19</u> , 73.
Harde	(1921)	C. R. Soc. Biol. (Paris) <u>84</u> , 968.
Hardenbergh, J. G.	(1926)	J. Amer. vet. med. Ass. <u>68</u> , 309.
Hathaway, B. M.	1935	Arch. Path. <u>19</u> , 819.

- |                                                      |       |                                                                     |
|------------------------------------------------------|-------|---------------------------------------------------------------------|
| Hayflick, L.                                         | 1961  | Exp. Cell Res. <u>23</u> , 1.                                       |
| Hecht, V.                                            | 1910  | Cited by Robert & Bains                                             |
| Hektoen, L.                                          | 1905  | J. infect. Dis. <u>2</u> , 238.                                     |
| Henning, M.W.                                        | 1956  | Animal Diseases in S.A.<br>3rd Edn. Central news<br>agency, p. 940. |
| Hilleman, M.R. & Werner, J.H.                        | 1954  | Proc. Soc. exp. Biol.<br>(N.Y.) <u>85</u> , 183.                    |
| Ho, M. & Enders, J.F.                                | 1959  | Proc. nat. Acad. Sci.<br>(Wash) <u>45</u> , 385.                    |
| Hoekenga, M.T., Schwarz,<br>A.J.F., & Palma, H.C.    | 1960  | J. Amer. med. Ass.<br><u>173</u> , 868.                             |
| Home, F.                                             | 1759  | Medical Facts & Figures<br>Kincaid & Bell J. Edin.                  |
| Hopper P.K.                                          | 1959a | Acta. paediat. <u>48</u> , 43.                                      |
| Hopper, P.K.                                         | 1959b | J. comp. Path. <u>69</u> , 78.                                      |
| Horne, R.W. & Nagington, J.                          | 1959  | J. mol. Biol. <u>1</u> , 333.                                       |
| Horne, R.W. & Waterson, A.P.                         | 1960  | IBID <u>2</u> , 75.                                                 |
| Horne, R.W. & Wildy, P.                              | 1962  | Brit. med. Bull. <u>18</u> ,<br>199.                                |
| Hsuing, G.D., Mannini A.<br>& Melnick, J.L.          | 1958  | Proc. Soc. exp. Biol.<br>(N.Y.) <u>98</u> , 68.                     |
| Hsuing, & Melnick, J.L.                              | 1958  | J. Immunol, <u>80</u> , 282.                                        |
| Imagawa, D.T. & Adams, J.M.                          | 1958  | Fed. Proc. <u>17</u> , 441.                                         |
| Imagawa, Yoshimori, M.,<br>Wright, S.W. & Adams J.M. | 1954  | Proc. Soc. exp. Biol.<br>(N.Y.) <u>87</u> , 2.                      |

- Imagawa, D. T., Yoshimori, M 1953 Bact. Proc. p. 50  
& Adams, J. M.
- Issacs, A., & Lindenman 1957 Proc. roy. soc. B.  
147, 258.
- Issacs, A. 1962 Brit. med. J. p. 353
- Jenner, E. 1809 Med. churg. Tran.  
Vol. 2.
- Jensen, K. E. 1956 Amer. J. publ. Hlth.  
p. 254.
- Jensen, K. E., & Francis T. 1953 J. Immunol, 70, 321.
- Josias, A. 1898 quoted by Rivers &  
Horsefall (1959) ~~7~~
- Kalra, S. L. & Rao, K. N. A. 1949 Indian J. med. Res. 37, 373,  
395.
- Karzon, D. T. 1955 Pediatrics, 16, 809
- Karzon, D. T., & Bussel, R. H. 1959 Science, 130, 1708
- Katz, S. L., Milanovic, M. U. 1958 Proc. Soc. expl. Biol.  
(N. Y.) 97, 23.
- Kohn, B. L. & Koiransky, H. 1933 Amer. J. Dis. Child,  
46, 40.
- Kreider 1943 The bacteriology &  
Pathology & etiology  
of measles pneumonia.  
Springfield, Ill. p. 1
- Laidlaw, P. P. 1930 A system of Bacteri-  
ology M. R. C. 1, 232.

Laidlaw, P. P. & Dunkin G. W.	1926	J. comp. Path. <u>39</u> , 222.
Laidlaw, P. P. & Dunkin G. W.	1927	IBID <u>41</u> , 209.
Lavin N. M.	1954	Nature (London) <u>173</u> , 174
Lavin N. M. & Hodgman S. F. A.	1954	Vet. Rec. <u>66</u> , 339.
Lasfargues, E. V. & Lasfargues J.	1952	C. R. Acad. Sci. (Paris) <u>146</u> , 1711
Ligniers, J.	1906	abstracted by Henning (1956)
Lendrieu A. C.	1947	J. path. Bact. <u>49</u> , 590.
Likar & Wilson	1959	Brit. J. exp. Med. <u>40</u> , 149.
Liu chien & Coffin D. L.	1957	Virology, <u>3</u> , 115.
Loeb M. R. & Lichtenstein J.	1958	Proc. nat. Acad. Sci. (Wash), <u>44</u> , 1004.
Lozovskaia	1959	quoted by Cutchins & Day huff (1962)
Lucas, W. P. & Prizer, E. L.	1912	J. med. Res. <u>26</u> , 181.
MaCallum	1919	Monograph <u>10</u> , Rockefeller institute of Medical Research.
Machlowitz, R. A., Spicer, D. S., Biynack, E. B. & Tyfell, A. A.	1961	Proc. Soc. expl. Biol. (N. Y.) <u>108</u> , 508.
MacIntyre, G. B., Trevan D. J. & Montgomerie, R. F.	1948a	Vet. Rec. <u>60</u> , 635.
MacIntyre, G. B. & Trevan, D. J.	1948b	Vet. Bull. <u>19</u> , 401.



12

Mackie & McCartney	1960	Handbook of Bacteriology, Ed. R. Cruickshank, Livingstone, Edin.
Mansi, W.	1957	J. comp. Path. <u>67</u> , 297
Mansi, W.	1961	Vet. Rec. <u>73</u> , 1102.
Mayer, M.N., Osler, A.G., Bier, O.G. & Heidelberger, M.	1946	J. exp. Med. <u>84</u> , 535.
Mo, J.L. De & Gover, T.A.	1961	Virology, <u>13</u> , 367.
Meyer, E. De.	1960	IBID <u>II</u> , 634.
Meyer, H.M. jr., Brookes, B. E., Douglas, R.D. & Rogers, N.G.	1962	Amer. J. Dis. child, <u>103</u> , 307.
Milanovic, M. V., Enders, J.F. & Mitus, A.	1957	Proc. Soc. exp. Biol. (N. Y.) <u>95</u> , 120.
Mitus, A.	1962	Amer. J. Dis. Child <u>103</u> , 331.
Mitus, A., Enders, J.F., Craig, J.M., & Halloway, A.	1959	New. Eng. J. Med. <u>261</u> , 882.
Morse, H.G. Chow, T. L. & Brandly, C.A.	1953	Proc. Soc. exp. Biol. (N. Y) <u>84</u> , 10.
Moulton, J.E. & Brown, & McCarthy K. McCarthy K., Mitus, A. Cheatham, W., & Peebles, T.	1954 1958	IBID <u>86</u> , 99. Amer. J. Disc. child. <u>96</u> , 500.
McCarthy, K. & Antigen	1956	
Mcgowan, J.P.	1911	J. Path. Bact. <u>15</u> , 372
Moore, R.A. & Gross, P.	1930	Amer. J. Dis. child. <u>40</u> , 247.

- Musser, S. J. & Underwood, G. E. 1960 J. Immunol. 85, 292
- Nagington, J. & Horne, R. W. (1962) Virology, 16, 248.
- Nicolle, C. 1931 Arch. Inst. Pasteur, Tunis, 20, 321
- Nevin N. & Bettman, F. R. 1921 J. inf. Disc. 29, 429.
- Nevin N. & Bettmen, F. R. 1923 IBID 30, 33.
- Oddo, F. G., Flaccamio, R. 1961 Virology, 13, 550.  
and Sinatra.,
- Osterhout, S. & Tamm, I. 1959 J. Immunol, 83, 442.
- Palmer, C. R. & Black, F. L. 1961 Proc. Soc. exp. Biol. (N. Y.), 107, 588.
- Pannel, L. & Cordle, M. S. 1960 Stain Tech.,
- Panum, P. L. 1846 quoted by Rivers (1959)
- Papp, K. 1937 Bull. Acad. nat. Med. (Paris), 117, 46.
- Papp, K. 1956 Rev. Immunol. (Paris), 20, 27.
- Paterson, J. S. 1957 The U. F. A. W. Hand-  
book on the care and  
management of Lab-  
oratory animals, 2nd  
Ed., U. F. A. W. Lond.,  
533.
- Parker, R. C. 1961 Methods of Tissue  
Culture, 3rd Ed.,  
Pitman Medical Pub-  
lishing Co. Ltd., Lond.,

- Paul, J. 1960 Cell and Tissue Culture  
2nd Ed., E. & S.  
Livingstone, Ltd.,  
Edinburgh & Lond.
- Peebles, T.C., McCarthy, K. 1957 J. Immunol., 78, 63.  
Enders, J.F. & Halloway, A.
- Peries, J.R. & Chany, C. 1960 C.R. Acad. Sci. (Paris)  
251, 820.
- Phisalix, 1903 IBID 55, 921
- Piercy, S.P. 1962 Vet. Rec. 74, 590
- Pinkerton, H., Smiley, W.L. 1945 Amer. J. Path., 21, 1  
& Anderson, W.A.D.
- Platz, H. 1938 Bull. Acad. Méd. (Paris)  
119, 598.
- Plowright, W. & Ferris, R.D. 1959a J. comp. Path., 69,  
152.
- Plowright, W. & Ferris, R.D. 1959b Nature, Lond., 179,  
316
- Plummer, P.J.G. 1939 Canad. J. comp. Med.,  
3, 96.
- Pollard, M. & Starr, Th.J. 1962 Progress in Medical  
Virology, Karger/  
Basel 4, 54.
- Puck, T.T., Cieciura, S.J. & 1957 J. exp. Med., 106,  
Ficher, H.W. 145.
- Puntoni, 1923 cited by Green &  
Carlson (1945)
- Puntoni, 1924 quoted by Green &  
Carlson (1945)
- Rabb, 1900 quoted by Cutchins &  
Dayhuff (1962)

Rake, G.	1959	Viral & Rickettsial Diseases of Man 3rd Ed., edit. Rivers & Horsefall, published by Pitman, Lond., p. 741.
Rake, G. & Schaffer, M. F.	1939	Nature, (Lond.) <u>144</u> , 672.
Rake, G. & Schaffer, M. F.	1940	J. Immunol., <u>38</u> , 177.
Rake, G. & Schaffer, M. F.	1941	J. infect. Dis., <u>69</u> , 65.
Rappaport, C. A.	1956	Bull. Wld. Hlth. Org., <u>14</u> , 147.
Rapp, F.	1960	Virology, <u>10</u> , 86.
Rapp, F. & Seligman, S. J. Jaross, L. B. & Gordon, I.	1959	Proc. Soc. exp. Biol. (N. Y.), <u>101</u> , 289.
Rapp, F., Gordon, I. & Baker, R. F.	1960	J. biophys. biochem. Cytol., <u>7</u> , 43.
Ray, B. G. & Swain, R. H. A.	1954	J. Path. Bact., <u>67</u> , 247.
Reagan, R. L. & Bruechner, A. L.	1951	Cornell Vet., <u>41</u> , 141
Recoulard, P., Nicol, L., Girard, O., Corvazier, R. Cheyrous, M. & Sizaret, P.	1960	Ann. Inst. Pasteur, <u>98</u> , 344.
Reed, L. J. & Muench, H.	1938	Amer. J. Hyg., <u>27</u> , 493
Reid, D. D.	1958a	Lancet, <u>1</u> , 1237.
Reid, D. D.	1958b	IBID, <u>1</u> , 1289.
Reissig, M., Black, F. L. & Melnick, J. L.	1956	Virology, <u>2</u> , 836.
Richardson, G. M.	1941	Lancet, <u>2</u> , 696.
Riordan, J. T., Ledinko, N. & Melnick, J. L.	1952	Amer. J. Hyg., <u>55</u> , 339.



- Rivers, T.M., Haagen, E. 1929 J. exp. Med., 50, 665.  
& Muckenfuss, R.S.
- Rivers, T.M. & 1959 Viral & Rickettsial  
Horsefall, F.L. Diseases of Man, 3rd  
Ed., Pitman, Lond.
- Robbins, F.C. 1962 Amer. J. Dis. Child.,  
103, 266.
- Robbins, F.C., Enders, J.F. 1950 Proc. Soc. exp. Biol.  
& Weller, T.H. (N.Y.), 75, 370.
- Roberts, G.B.S. & Bain, A.D. 1958 J. Path. Bact., 76, 111
- Rockborn, G. 1958a Arch. ges. Virusforsch.  
8, 485.
- Rockborn, G. 1958b IBID, 8, 493.
- Rockborn, G. 1958c IBID, 8, 500.
- Rockborn, G. 1959 Nature, Lond., 184,  
822.
- Rockborn, G. 1960 J. small Animal Prac-  
tice, 1, 94.
- Rockefeller, 1959 Rockefeller Annual  
Report.
- Rolleston, H. 1924 Brit. med. J., 2, 917
- Roizman, B. 1961 Virology, 13, 387.
- Roizman, B. & Schlunderberg, 1961 Proc. Soc. exp. Biol.  
A. E. (N.Y.), 106, 320.
- Rosanoff, E.I. 1961 IBID, 106, 536.
- Rosen, L. 1960 Amer. J. Hyg., 71,  
120.
- Rosen, L. 1961 Virology, 13, 139.

Ruckle, G.	1957a	J. Immunol., <u>78</u> , 330.
Ruckle, G.	1957b	IBID, <u>79</u> , 361
Ruckle, G.	1962	Amer. J. Dis. Child., <u>103</u> , 297.
Ruckle, G. & Rogers, K.D.	1957	J. Immunol., <u>78</u> , 341.
Ryazantseva, N.E.	1956	Excerpta med., (Amst), Sect. IV, <u>10</u> , abstract no. 2160.
Salzman, N.P.	1960	Virology, <u>10</u> , 150.
Saver, R.M. & Fegley, H.C.	1960	Ann. N.Y. Acad. Sci. <u>85</u> , 866.
Schaffer, M.F., Rake, G. & Hodes, H.L.	1942	Amer. J. Dis. Child., <u>64</u> , 815.
Schaffer, M.F., Rake, G. & Stokes.	1941	J. Immunol., <u>41</u> , 241
Schluederberg, A.E. & Roizman, B.	1962	Virology, <u>16</u> , 80.
Schroeder, H.	1929	cited by Henning (1956)
Schwartz A.J.F. & Zirzbel, L.W.	1959	Proc. Soc. exp. Biol. (N.Y.), <u>102</u> , 711.
Schwartz, A.J.F., Boyer, P.A., Zirzbel, L.W. & York, C.J.	1960	J. Amer. med. Ass., <u>173</u> , 861.
Schwartz, J.F. & Zirzbel, L.W.	1960	Proc. Soc. exp. Biol. (N.Y.), <u>104</u> , 526.
Scott, G.R. & Brown, R.D.	1961	Bull. epiz. Dis. Afr., <u>9</u> , 83.
Seligman, S.J. & Rapp, F.	1959	Virology, <u>9</u> , 143.

- Semsworth, K.H. 1939 Arch. Path., 28, 386.
- Sergiev, P.G., Ryazantseva, N.E. & Smironova, 1956 Excerpta med. (Amst.) Sect. IV, 10, abstract no. 2395.
- Sergiev, P.G., Ryazantseva, N.E. & Schroit, I.G. 1960 Acta. Virol. (Engl.), 4, 265.
- Scherman, F.E. & Ruckle, G. 1958 Arch. Path. 65, 587.
- Slanetz, C.A. & Smetana, H. 1937 J. exp. Med., 66, 655.
- Sidertoff, H.A. & Green, R.G. 1942 J. infect. Dis., 71, 253.
- Simon, M.A. & Ballon, H.C. 1948 Amer. J. clin Path., 18, 796.
- Skaggs, J.W. 1960 J. Amer. vet. med Ass., 130, 8.
- Skinner, H. & Bradish, C.J. 1954 J. gen. Microbiol. 10, 377.
- Stone, S.S. 1960 Virology, 11, 638.
- Tiniguchi, T., Okuno, Y. 1956 Med. J. Osaka, Univ. 6, 1013.
- Aoyama, A., & Kusumoto, K.
- Tawara, J.T., Goodman, J.R. 1960 Fed. Proc. 19, No. 1
- Imagawa, D.T. & Adams, J.M.
- Toolan, H.W. 1954 Cancer Res. 14, 660.
- Tompkin, U. & Macaulay, J.C. 1955 J. Amer. med. Ass. 157, 711.
- Torres, C.M. & Teixeira J. Le C. 1935 C.R. Soc. Biol. (Paris) 118, 908.
- Torrey, J.C. & Rake, A.H. 1913 J. med. Res. 27, 291.
- Toyoshima, K. Takalashi, M. 1959 Bikens. J. 2, 305.
- Kunita, N., & Okunoy.

- Toyoshima, K. Fukai, K. 1960 IBID 3, 133.  
Hata, S. Kunita, N.
- Toyoshima, K., Hata, S., 1960 IBID 3, 241.  
Takalashi, M., Miki, T., &  
Okuno, Y.
- Ullua 1735- quoted by Henning  
1746
- Underwood, G.E. 1959 J. Immunol. 83, 198.
- Vantsis, J. T. 1959 Vet. Rec. 71, 99.
- Verlinde, J.D. 1948 quoted by Henning (1956)
- Warren, J. 1960 Advanc. Virus Res. 7,  
27.
- Warren, J. & Cutchins, E.C. 1957 Virology, 4, 207.
- Warren, J., Nadel, M.K., 1960 Amer. J. vet. Res.  
Slater, E. & Millian, S.J. 21, 111.
- Warthin 1931 Arch. Path. (Chicago)  
11, 864.
- Waterson, A.P. Cruickshank, 1961 Virology, 15, 379.  
J.G. ; Lawrence G.D.  
Kanaret, A.D.
- West, F.K.
- West, J.L. & Brandly, C.A. 1955 Cornell. Vet. 45, 560.
- White, G. & Cowan, K.N. 1962 Virology, 16, 209.
- White, G., Simpson, R.M., 1961 Immunol. 4, 203.  
& Scott, G.R.



Whitney, L. F.	1943	Vet. Med., <u>38</u> , 419.
Winslow, C. E. A.	1943	Conquest of Diseases, Princeton, U. Press. 267-274.
Wright, J.	1957	Lancet, <u>96</u> , 667.
Youngner, J. S.	1954	Proc. Soc. exp. Biol. (N. Y.) <u>85</u> , 202.
Zitcer, E. M.    Fogh, T. & Dunnebac, T. H.	1955	Science <u>122</u> , 30.